Antioxidant as potential immunoadjuvant in anti-tuberculosis immunotherapy

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by

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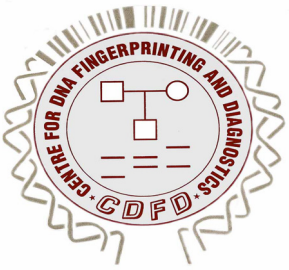

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DECLARATION

The research work presented in this thesis entitled **"Antioxidant as potential immunoadjuvant in anti-tuberculosis immunotherapy"**, has been carried out by me at the Centre for DNA Fingerprinting and Diagnostics, Hyderabad, India under the guidance of Dr. Sangita Mukhopadhyay. I, hereby, declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

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CERTIFICATE

This is to certify that thesis entitled, **"Antioxidant as potential immunoadjuvant in anti-tuberculosis immunotherapy",** submitted by Mr. Kaiser Alam for degree of Doctor of Philosophy to Manipal University is based on the work carried out by him at the Center for DNA Fingerprinting and Diagnostics, Hyderabad, India. This work is original and has not been submitted in part or full for any degree or diploma of any other university or institution.

Dr. Sangita Mukhopadhyay — Thesis Supervisor

Dr. Shekhar C. Mande — Dean Academic Affairs

Dedicated to .....

My parents

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Contents

ABBREVIATIONS

°C	--- degree centigrade
AP-1	--- activator protein-1
APCs	--- antigen presenting cells
BCG	--- bacillus Calmette-Guerin
BSO	--- L-burhionine(S,R)-sulphoximine
Btk	--- bruton's tyrosine kinase
CaM	--- calmodulin
CE	--- cytoplasmic extract
DCFH	--- 2', 7'-dichlorofluorescein diacetate
DCs	--- dendritic cells
DMF	--- dimethlylformamide
DNA	--- deoxyribonucleic acid
DNp38	--- dominant negative mutant of p38
DTT	--- dithiothreitol
E.coli	--- *Escherichia coli*
EIA	--- enzyme-linked Immunoassay
EMSA	--- electrophoretic mobility shift assay
FACS	--- fluorescence-activated cell sorting
FBS	--- fetal bovine serum
GSH	--- reduced glutathione
GSH-OEt	--- glutathione ethyl ester reduced
GSSG	--- oxidized glutathione
GST	--- glutathione *S*-transferase
H_2O_2	--- hydrogen peroxide
HRP	--- horseradish peroxidase
HSD	--- honestly significant difference
hsp60	--- heat shock protein 60
IB	--- immunoblotting
ICAM	--- intracellular adhesion molecule
IFN-γ	--- interferon-gamma
IKK	--- I-kappa B kinase
IL	--- interleukin
IPTG	--- Isopropyl β-D-1-thiogalactopyranoside
IκBα	--- inhibitory subunit of kappa B
ΔIκBα	--- phosphorylation-defective IκBα
iNOS	--- inducible nitric oxide synthase
IP	--- immunoprecipitation
kDa	--- kilodaltons
LB	--- Luria-Bertani
LPS	--- lipopolysaccharide
MDM	--- monocyte-derived macrophages
mg	--- milligram
ml	--- mililitre
mM	--- millimolar

ABBREVIATIONS

Mtb	--- *M. tuberculosis*
MTT	--- 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NAC	--- N-acetyl-L-cysteine
NaF	--- Sodium fluoride
NE	--- nuclear extract
NFAT	--- nuclear transcription factor of activated T cells
NF-Kb	--- nuclear transcription factor kappa B
Ni-NTA	--- Ni-nitrilotriacetic acid
NO	--- nitric oxide
OD	--- optical density
OPD	--- o-phenylenediamine tetrahydrochloride
PBMC	--- peripheral blood mononuclear cells
PBS	--- phosphate buffer saline
PECs	--- peritoneal exudate cells
PMA	--- phorbol 12-myristate 13-acetate
PMSF	--- phenyl methyl sulphonyl fluoride
pp38	--- phospho p38
PPD	--- purified protein derivative
RNA	--- ribonucleic acid
ROI	--- reactive oxygen intermediate
ROS	--- reactive oxygen species
rpm	--- rotations per minute
RT-PCR	--- reverse transcription polymerase chain reaction
siRNA	--- small interfering RNA
SOCS1	--- suppressor of cytokine signaling 1
SDS-PAGE	--- sodium dodecyl sulfate polyacrylamide gel electrophoresis
TB	--- tuberculosis
TFP	--- trifluoperazine
Th	--- T-helper
TLR	--- toll like receptor
TNF	--- tumor necrosis factor

Chapter 1 - Introduction and Review of literature

1.1. Macrophage and immune responses

The activation of macrophages is one of the first events in the innate resistance to intracellular infection. Activation of macrophages during infection with intracellular pathogens results in production of a series of proinflammatory cytokines which further contribute to phagocytic cell activation and induction of various inflammatory responses. These non-antigenic immune responses, in many cases, are very effective in eliminating pathogens or at least reducing significantly their ability to multiply. Some of the cytokines released by the macrophages and other inflammatory cells have a direct anti-pathogen activity or contribute to the activation of effector cells of innate immunity such as natural killer (NK) cells. For example interferon-alpha (IFN-α) and IFN-β, both of which have anti-viral activity and enhance cytotoxic activity of NK cells (Scott and Trinchieri, 1995). However, complete elimination of pathogen is mediated by antigen specific adaptive immunity viz. activated T and B cells. The mechanism of innate resistance and adaptive immunity, however, are interdependent and cross regulate each other during infection (Trinchieri et al., 1993). Membrane protein interactions and soluble factors, such as cytokines, antibodies, complement, and their receptors on the cells, represent the elements of communication of the intimate cross-talk between the two brands of resistance against pathogens. The inflammatory responses of innate resistance set the stage for T cell differentiation by influencing the cytokine profile in which antigen specific T cells expand in response to antigenic peptides exposed on the

antigen presenting cells (APCs). Depending on cytokines present during T-helper (Th) cell differentiation, the T cells either differentiate into Th1 or Th2 cells. For example induction of higher levels of IL-12 and tumor necrosis factor-alpha (TNF-α) activates development of Th1-type T cell response ((Hsieh et al., 1993; Mosmann et al., 1986; Trinchieri, G., 1998) whereas an increased production of IL-10 or nitric oxide favors a Th2-type T cells (D'Andrea et al., 1993; Mukhopadhyay et al., 1999; Mukhopadhyay et al., 2006). Differential expressions of cytokines such as IL-12 and IL-10 by macrophages are known to dictate the development of Th1 and Th2 T cell responses. The Th1 T cells produce interleukin-2 (IL-2) and interferon-gamma (IFN-γ) and favor cell-mediated responses and macrophage activation, effective in the resistance to intracellular pathogens (Mukhopadhyay et al., 2006). Whereas the Th2 T cells, important for mediating immunity against the extracellular parasites produce IL-4, IL-5, IL-6 and IL-10 and favor humoral immunity and allergic responses. A balance in Th1/Th2 is found to be crucial for inducing effective host immune response to infection.

1.2. Glutathione

Glutathione is ubiquitously present in cells and is enzymatically formed by glutamic acid, glycine and cysteine. It is the most abundant tripeptide and the most prevalent cellular thiol inside the eukaryotic cells (Deneke and Fanburg, 1989). Glutathione is 85–90% freely distributed in the cytosol, but can also be compartmentalized in organelles

including the mitochondria, the peroxisomes, the nuclear matrix, and the endoplasmic reticulum (ER) after its cytosolic synthesis. It serves as a fundamental antioxidant defense mechanism against free radicals, electrophilic compounds, xenobiotics and other oxidant (Meister and Anderson, 1983). Glutathione is known to function directly or indirectly in many important biological phenomenons including the synthesis of protein and DNA, transport, enzyme activity, metabolism and protection of cells (Meister and Anderson, 1983). In addition, glutathione is a co-enzyme for several enzymatic reactions and participates in their metabolism (Meister and Anderson, 1983). The role of glutathione has also been implicated in various cellular events such as inflammation, regulation of redox-regulated signal transduction, regulation of cell proliferation, remodeling of extracellular matrix, apoptosis and mitochondrial respiration (Rahman, 2005). Glutathione plays an important role to maintain the functional integrity of a physiologically active system (Haddad and Harb, 2005). Depletion of glutathione has been linked to pathophysiological conditions in many diseases (Bunnell and Pacht, 1993; Cantin et al., 1989; Saugstad, 1997).

Recent evidence indicates that a change in glutathione milieu can lead to progression of apoptotic cell death (Pervaiz and Clement, 2002). Glutathione depletion is a common feature of apoptotic cell death triggered by a wide range of stimuli including activation of death receptors, stress, environmental agents, and cytotoxic drugs. Although initial studies indicate that depletion of glutathione is actually only a byproduct of oxidative stress generated during cell death, recent data suggest that glutathione depletion is a

critical regulator of apoptosis (Franco and Cidlowski, 2009). High intracellular GSH levels have been associated with apoptotic resistant phenotypes in several models of apoptosis, and glutathione depletion by itself has been observed to either induce or stimulate apoptosis. Interestingly, L-buthionine-(S,R)-sulphoximine (BSO)-induced glutathione depletion does not trigger apoptosis, but potentiates death receptor-induced apoptosis in T cells (Armstrong et al., 2002; Friesen et al., 2004). At present, the exact contribution of mitochondrial versus cytoplasmic glutathione pools in apoptosis is not fully understood. Some reports suggest that apoptosis correlates directly with depletion of cytosolic glutathione rather than with mitochondrial glutathione depletion (Will et al., 2002). However, other studies have shown that mitochondrial glutathione depletion is important in triggering the cell death cascades. Accordingly, activation of cell death by diseases or treatments that deplete cellular glutathione seems to correlate closely to the extent of depletion of mitochondrial glutathione rather than the changes in the cytoplasmic pool (Lash, 2006).

Glutathione is found to be essential for development. It has been shown that γ-glutamyl-cysteinyl synthetase (γ-GCS), (the rate limiting enzyme for glutathione synthesis) knock-out mice died due to apoptotic cell death (Shi et al., 2000). Glutathione is also involved in S-glutathionylation, a post-translational modification whereby the cysteine-sulfhydryl moiety of glutathione forms a disulfide bond with a cysteine-sulfhydryl moiety of a protein, and it represents a mechanism of reversible redox regulation of protein activity and cell signaling (Gallogly and Mieyal, 2007; Shelton et al.,

2005). In many cases, S-glutathionylation can inactivate some proteins like glyceraldehyde-3-phosphate dehydrogenase, protein tyrosine phosphatase and nuclear factor-1, although there are reports that S-gluathionylation can also activate some proteins like human immunodeficiency virus-1 protease and hRas (Shelton et al., 2005). Many diverse cellular proteins such as transcription factors, adhesion molecules, enzymes, and cytokines are reported to undergo glutathionylation, although it's functional impact has not been well characterized (Shelton and Mieyal, 2008).

The pathways involved in glutathione synthesis and it's metabolism is well characterized. The rate of glutathione synthesis is determined by availability of cysteine and the activity of γ-GCS. Glutathione is formed by the consecutive action of γ-GCS and glutathione synthetase, utilizing ATP as shown in Fig. 1.1 Both the enzymes are exclusively cytosolic (Rahman, 2005). The mammalian γ-GCS holoenzyme is heterodimeric consisting of a 73 kDa catalytic heavy chain and a 30 kDa modifying light chain that modulate the heavy chain for substrate and inhibitor (Huang et al., 1993). Although the rat heavy chain is represented by a single transcript, hybridization experiment shows two mRNAs coding for human heavy chain in most human tissues. There are two transcripts that are consistently seen in light chain in both rat and human (Gipp et al., 1995). About 30% to 70% isolated rat and human γ-GCS is stabilized by inter-chain disulfide bond. Because γ-GCS disulfide bond persist even in presence of thiol, it is clear that non-covalent forces also contribute in stabilization of dimer. Presence or absence of disulfide bond is thought to modulate affinity for substrate and

inhibitor. It has been shown that γ-GCS is regulated by various transcription factor binding sites such as activator protein-1 (AP-1) and antioxidant response element (ARE) in response to various environmental, oxidant, antioxidant and inflammatory stimuli (Rahman et al., 1998). The γ-GCS enzyme is inhibited non-allosterically by glutathione under conditions similar to those that prevail *in vivo*, indicating a physiological significant feedback mechanism (Richman and Meister, 1975).

Glutathione synthetase (GS) is the second enzyme in the glutathione biosynthesis pathway. It catalyses the condensation of γ-glutamyl-cysteine and glycine, to form glutathione. Mammalian GS is a homodimer with each subunit of 52 kDa containing about 2% carbohydrate (Oppenheimer et al., 1979). The rat enzyme has been cloned and sequenced. In mouse a single gene produces six distinct mRNAs, three of which code for the same enzyme, the other mRNAs encode truncated proteins with no established activity. GS does not contain any inter subunit disulfide bond and is stabilized by noncovalent forces (Shi et al., 1996). Glutathione peroxidase, first described as an enzyme that protects hemoglobin from oxidative degradation in red blood cells, is a family of selenium-containing enzyme. It converts H_2O_2 to H_2O by oxidizing reduced glutathione (GSH) as shown in Fig. 1.1. Glutathione peroxidase requires several secondary enzymes (glutathione reductase and glucose-6-phosphate dehydrogenase) and co-factors (reduced glutathione, NADPH and glucose 6-phosphate) to function at high efficiency. Several different isoforms of glutathione peroxidase have been identified with glutathione peroxidase 1 to be the major enzyme responsible for

removing H_2O_2. Glutathione peroxidase is involved to protect cells against oxidative damage (Mirault et al., 1991) and inhibits apoptosis induced by H_2O_2 (Kayanoki et al., 1996). It plays a role in the suppression of the malignant phenotype (Liu et al., 2004). The crystal structure of human plasma glutathione peroxidase was determined upto resolution of 2.9 Å. (Ren et al., 1997). The asymmetric unit contains a dimer. Tetramers are formed from dimers by crystallographic symmetry. The subunit structure of the plasma enzyme shows the typical structure motif of the thioredoxin fold consisting of a central β-sheet and several flanking α-helices. The active site selenocysteine residue is located in the loop between β1 and α1 and is located in a pocket on the protein surface (Ren et al., 2007).

Glutathione reductase is a widely distributed flavoprotein which catalyses conversion of GSSG to GSH, and was first identified in 1930. The reaction catalysed by glutathione reductase is irreversible and is responsible for high GSH/GSSG ratio found in the cells. Glutathione reductase has been identified from several sources and all of them are having higher amino acid homology. The three dimensional structure of human erythrocyte glutathione reductase was determined at 1.9 Å resolution (Savvides et al., 2002). This enzyme has two identical subunits, each of which has four domains and a flexible 18 amino acid residues at the N-terminal. The crystal structure of erythrocyte glutathione reductase has revealed that the NADPH and GSSG binding positions are on the opposite site of a subunit. Transfer of reducing equivalent are carried out by a ring of FAD located at the centre of each subunit as well as adjacent cysteine residue.

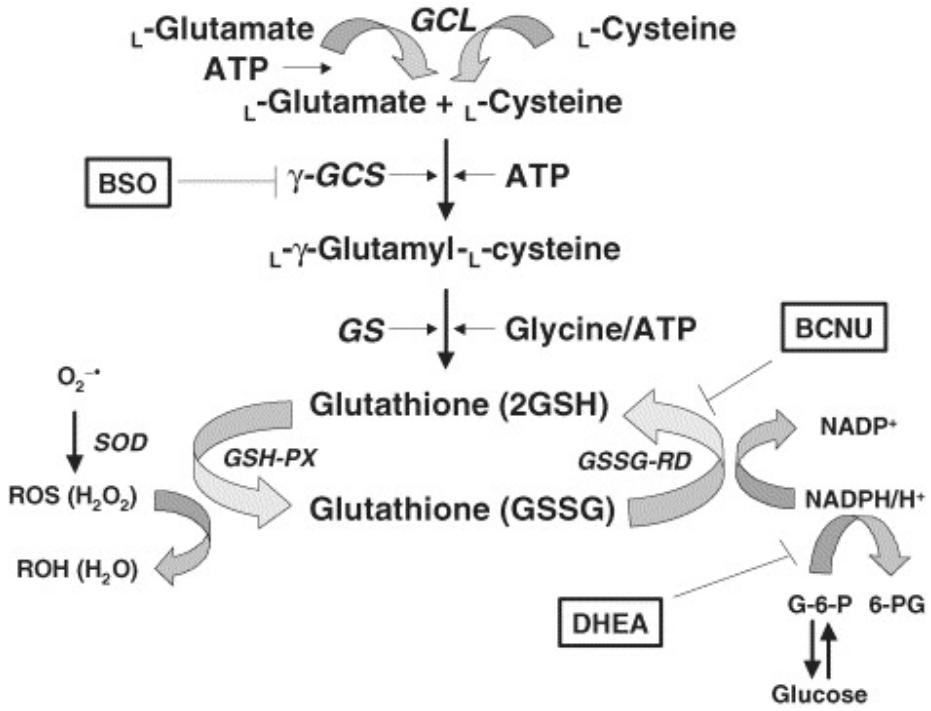

Figure 1.1. Diagram of the redox cycle showing the relationship between antioxidant enzymes and glutathione. Glutathione (GSH) is synthesized from amino acids by the action of γ-glutamyl-cysteinyl synthetase (γ-GCS), the rate-limiting enzyme, and glutathione synthetase (GS). This reaction requires energy, is ATP-limited and is specifically inhibited at the level of γ-GCS by L-buthionine-(S,R)-sulfoximine (BSO). GSH undergoes the glutathione-peroxidase (GSH-PX) coupled reaction, thereby detoxifying reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2). A major source of H_2O_2 is the biochemical conversion of superoxide anion (O_2•−) by the action of superoxide dismutase (SOD). During this reaction, GSH is oxidized to generate GSSG, which is recycled back to GSH by the action of glutathione reductase (GSSG-RD) at the expense of reduced nicotinamide (NADPH/H+), thus forming the redox cycle. The reduction of the glutathione pathway is blocked by the action of 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU). The major source of NADPH/H+ comes from the conversion of glucose, a reaction blocked by dehydroepiandrosterone (DHEA). (*Adapted from J.J. Haddad, H.L. Harb / Molecular Immunology 42 (2005) 987–1014)*

1.3. Glutathione and cellular redox state

Overcoming entropy is known to be required for the survival of living organism and is achieved by capturing the energy of oxidation processes of cells. The energy comes from the movement of electrons from oxidizable organic molecules to oxygen. Redox couples in cells are responsive to electron flow, i.e. changes in the reducing/oxidizing environment. There are many redox couples in the cell that work together. Some of these redox couples are linked to each other to form a set of related couples. The redox environment of a cell is a reflection of the state of these couples. Dr. Bücher and his group in 1953 were the first to estimate the actual cellular reduction potentials for the NAD^+/NADH and $NADP^+$/NADPH couples (Bucher, 1953). Redox state is a term that has been used to describe the ratio of the inter-convertible reduced and oxidized form of a specific redox couple. For example, Hans Krebs focused on the NAD^+/NADH couple and defined the redox state of this couple in a cell to be [free NAD^+]/[free NADH] (Krebs, 1967). In recent years, the term redox state has been used not only to describe the state of a particular redox pair, but also to describe the redox environment of a cell. The redox environment of a linked set of redox couples as found in a biological fluid, organelle, cell, or tissue is the summation of the products of the reduction potential and reducing capacity of the linked redox couples present (Schafer and Buettner, 2001) and indicated as:.

$$\text{Redox environment} = \sum_{i=1}^{n(couple)} E_i \text{ X } [\text{reduced species}]_i$$

Where, E_i is the half-cell reduction potential for a given redox pair and $[\text{reduced species}]_i$ is the concentration of the reduced species in that redox pair.

It may be difficult to measure all linked redox couples present in biological settings to determine the redox environment. Instead, a representative redox couple can be used as an indicator for changes in the redox environment. The determinants of redox environment inside the cells are glutathione balance represented as reduced glutathione (GSH)/oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate (NADP-/NADPH), and thioredoxin (TrxSH/trxSStrx) couples. Since the GSH concentration is 100 to 10000 fold higher than the reduced form of other couples, the major determinant of redox status in mammalian cells is glutathione (Rahman et al., 2005; Schafer and Buettner, 2001). However, the three redox systems are not isolated systems. Both the thioredoxin and GSH-systems use NADPH as a source of reducing equivalents; thus, they are thermodynamically connected to each other. NADPH is the thermodynamic driving force for the GSH and thioredoxin systems (Schafer and Buettner, 2001). Glutathione is considered to be the major thiol-disulfide redox buffer of the cell. On average, the GSH concentration in the cytosol is ranging from 1 mM to 11 mM (Smith et al., 1996). When dealing with homogeneous fluids such as plasma, the assessment of the redox environment is relatively uncomplicated because the

determination of the molar concentrations of GSH and GSSG is straightforward. But when dealing with cells or tissues, compartmentation of GSH and GSSG may pose a problem, as all compartments may be at a nonequilibrium steady-state with respect to each other. It is known that a measurement of total content of GSH and GSSG in cells would represent an overall redox environment. The ratio of GSH/GSSG in cytosol can vary from 10 to more than 1000 (http://www.nwlifescience.com/products/pdf/gsh01gssg.htm). The redox environment of the endoplasmic reticulum is found to be more oxidizing than the cytosol (Hwang et al., 1992). The more oxidizing environment of the endoplasmic reticulum appears to be needed to produce proteins that have necessary disulfide bonds. Thus, compartmentalization of GSH can result in different redox environments in various compartments when compared to that of the cytosol.

1.4. Role of glutathione-redox in cellular functions

Direction of many important and crucial functions of cells is heavily dependent on the cellular "redox-state". Glutathione redox plays a crucial role in maintaining equilibrium and establishing the mechanisms of cellular defenses (Haddad and Harb, 2005; Rahman, 2005). Glutathione-redox homeostasis is critical for numerous biological events to occur, such as enzyme activation, DNA synthesis, cell cycle regulation, transcriptional

activation of specific genes, and programmed cell death (Arrigo, 1999; Meister and Anderson, 1983)**.** Changes in the cellular redox environment can alter signal transduction, and regulation of the cell cycle (Arrigo, 1999). Activities such as ligand binding, DNA binding, and nuclear translocation have been shown to be under redox control (Makino et al., 1999; Simons and Pratt, 1995). Studies show that translocation of transcription factors to the nucleus is often redox-dependent (Okamoto et al., 1999).

Development of an advanced organism initiate with the division of a single cell. The progeny of this cell then turn into a multi-cell, multi-tissue, and multi-functional entity. This development has been shown to occur in a relatively reducing environment. In prenatal tissue GSH levels are found to be relatively high while antioxidant enzyme levels of SOD, GSH-PX, and catalase are low, consistent with a reducing environment (Allen and Balin, 1989). During the last few days in utero, rabbit lung-SOD and catalase levels increase 2-fold; GSH-PX increases 4-fold and GSH appears to decrease. These changes are probably for the preparations for higher oxygen levels to be encountered after birth. But they also suggest a general change to a more oxidized glutathione-redox environment. A clear example of the changes in redox environment during differentiation is provided by Allen et al. (1985) in a study using the slime mold (*Physarum polycephalum* (Allen et al., 1985). A sequential change in the antioxidant profile was observed upon providing a stimulus for differentiation. First, a substantial decrease (about 75%) in GSH was observed followed by a 7-fold increase in MnSOD. This

increase appeared before the progression to a differentiated form was observed morphologically. More recent data indicate that intracellular GSH levels fluctuate during the cell cycle (Shaw and Chou, 1986). Li and Oberley (1998) suggest that changes in the redox environment are necessary for a cell to successfully progress through the cell cycle (Li and Oberley, 1998). They found that during the M phase of the cell cycle of NIH/3T3 cells, total glutathione and glutathione disulfide levels were greatly increased as compared to the quiescent cells and cells in S phase. Also, Atzori et al. (1990) have reported that in tissue culture system, after the seeding of cells, GSH levels increase during the lag phase, get upregulated during the initial exponential growth phase, and then fall as cells become confluent (Atzori et al., 1990).

The role of GSH has also been implicated in various diseases. *In vitro* studies with HIV infection indicate that HIV infection induces a significant decrease in intracellular reduced glutathione (GSH) in human macrophages. Exogenous GSH strongly suppressed the production of p24 gag protein as well as the virus infectivity (Garaci et al., 1997). *In vitro* studies also suggest a link between GSH depletion and HIV disease progression because low GSH levels can promote HIV expression and can impair T cell immune function. Another study has demonstrated that *M. tuberculosis* grown *in vitro* is also sensitive to glutathione and its derivative *S*-nitrosoglutathione, and that glutathione is essential for the control of the intracellular growth of *M. tuberculosis* (Venketaraman et al., 2005).

The evidences are overwhelming that the glutathione has a vital role in the modulation of inflammatory responses. Augmentation of GSH level has been attempted by intravenous administration of GSH, oral ingestion of GSH and aerosol/inhalation of nebulized GSH to reduce inflammatory lung diseases (Borok et al., 1991; Ruhl and Erckenbrecht, 1990). GSH aerosol therapy normalized low GSH levels in the lungs in these patients; however, nebulised GSH can show a detrimental effect in the asthamatic patients by producing broncho-constriction presumably due to formation of GSSG (Marrades et al., 1997). This suggests that nebulised aerosol therapy is not appropriate way of increasing GSH levels in lung and cells in patients with asthama. Treatment of glutathione deficiency by administration of GSH is found to be an alternate approach to treat patients with chronic lung diseases. However it should be noted that cellular protection is likely to depend not only on cellular levels of GSH at the time of challenge, but on the capacity of cell to synthesize GSH and transport process that control substrate availability. The normal intracellular concentration of GSH cannot be affected by administration of exogenous GSH and peptide precursor because of feedback inhibition of γ-GCS by GSH. Better understanding at molecular and cellular levels of glutathione regulation is required to effectively treat various diseases. Understanding the glutathione-redox signaling mechanism can play a critical role in the development of pharmacological approaches to treat these diseases.

1.5. Interleukin (IL)-12

IL-12 was identified in 1989, as a soluble factor that could stimulate natural killer (NK) cells to produce IFN-γ (Mosmann et al., 1986). The cells that mainly produce IL-12 are macrophages, monocytes, neutrophils and dendritic cells in response to pathogen and other endogenous stimuli. Products from microorganisms such as bacteria, intracellular parasites, fungi, double-stranded RNA, bacterial DNA and CpG-containing oligonucleotides are strong inducers of IL-12 production through toll-like receptors (TLRs) or other receptors (D'Andrea et al., 1992). IFN-γ activated phagocytic cells are also primed to produce higher levels of IL-12 in a powerful positive feedback mechanism (Kubin et al., 1994). In contrast, a few microorganisms appear to inhibit IL-12 synthesis. HIV was the first example of a pathogen that selectively inhibits IL-12 synthesis. Other microorganisms such as *L. major* and *L. donovoni* have been shown to inhibit IL-12 synthesis without affecting IL-1, TNF-α, inducible nitric oxide synthase (iNOS) or IL-10 synthesis (Reiner et al., 1994). Inhibition of IL-12 may be an important strategy adopted by the pathogens to favor its long term survival and persistence inside the host.

The IL-12 cytokine has a unique molecular structure of a covalently linked heterodimer composed of two chains, p40 and p35 (Trinchieri et al., 2003) and both chains are covalently linked. The sequence of p35 is homologous to that of IL-6 and Granulocyte colony-stimulating factor (G-CSF) and structurally it has four α-helix bundles, typical of cytokines. The sequence of the p40 chain is homologous to the extracellular portion of

members of the hemopoietin receptor family, particularly the IL-6 receptor α chain (IL-6Rα) and ciliary neutrotrophic factor receptor (CNTFR) (Fig. 1.2). Messenger RNA encoding IL-12 p35 is present in many cell types including lymphocytes that do not produce IL-12 although it has never been detected in cell free culture supernatant by radioimmunoassay. This indicates the fact that IL-12 p35 is ubiquitously expressed but not secreted alone. By contrast, mRNA encoding IL-12 p40 is restricted to the cell that produces biologically active IL-12 (D'Andrea et al., 1992). However, IL-12 p40 is often secreted in large excess over the p70 heterodimer; p35 is only secreted as a part of the heterodimer when p40 is also produced by the same cells. Receptors for IL-12 (IL-12Rβ1 and IL-12Rβ2) are within the gp130 family of receptors (Presky et al., 1996). IL-12Rβ1 binds to IL-12 p40, and it is associated with TYK2, whereas IL-12Rβ2 recognizes either the heterodimer or the p35 chain and is associated with janus kinase 2 (JAK2). IL-12Rβ1 and IL-12Rβ2 activate the janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathways. The predominant response and most of the biological responses to IL-12 are mediated by STAT4 as indicated by the fact that the STAT4-deficient mice have identical phenotypes to mice that are deficient for IL-12 p40 (Thierfelder et al., 1996).

IL-12 p40 was also shown to associate with a p19 chain to form another novel covalently linked heterodimeric cytokine, IL-23. The p19, like p35, has homology with IL-6 and G-CSF, and is secreted only when is associated with IL-12 p40 (Oppmann et al., 2000). The IL-23 receptor complex is composed of IL-Rβ1 and a novel gp130-like chain, the IL-23R

(Parham et al., 2002). Similar to IL-12, IL-23 signaling also activates TYK2, JAK2, and STAT 1, 3, 4. However, the activation of STAT4 is not as predominant as IL-12, and STAT3/4 heterodimers, rather than STAT4 homodimers, are induced by IL-23. IL-23 is produced by similar cell types as IL-12, and the receptor complex is expressed or upregulated on T cells and NK cells, as well as phagocytic and dendritic hematopoietic cells. IL-23 has been proposed to have similar but not overlapping functions with IL-12 in inducing IFN-γ production, Th1 T cell differentiation, and activation of the antigen-presenting functions of dendritic cells (Parham et al., 2002). IL-12 is a relatively poor T cell mitogen and preferentially affects naive T cells whereas IL-23 selectively induces proliferation of memory T cells (Oppmann et al., 2000).

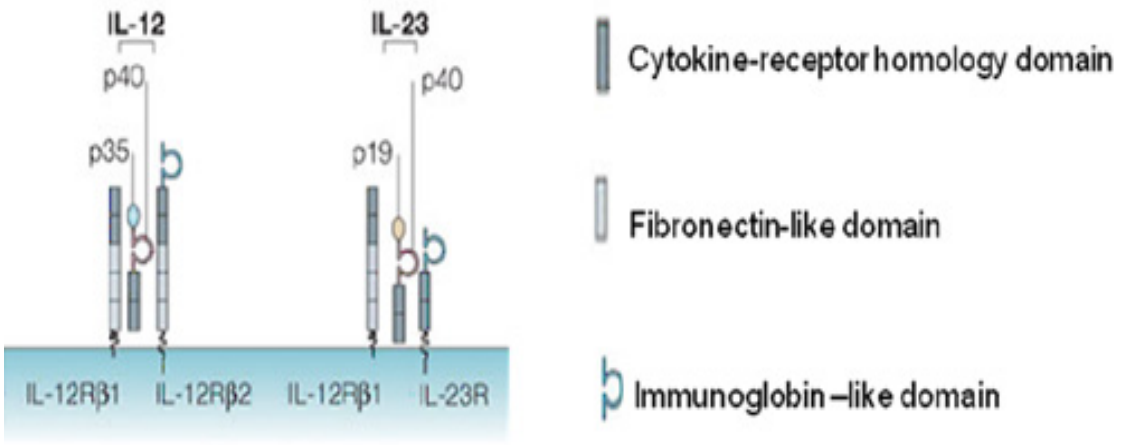

Figure 1.2. IL-12 is a covalently linked heterodimer composed of light chain IL-12 p35 and heavy chain IL-12 p40. The IL-12 receptor is comprised of IL-12 Rβ1 and IL-12Rβ2

1.6. Transcriptional control of IL-12 p40

Although the promoter of the gene encoding the IL-12 p40 has been studied in detail, sufficient information is not available for the gene encoding the p35. A close analysis of nucleosome positioning, chromatin remodelling and transcription-factor binding studies indicate that the p40 gene promoter in macrophages is normally configured in a nucleosome array (Fig. 1.3) (Weinmann et al., 2001). After activation of macrophages with the bacterial LPS, nucleosome is selectively remodelled such that it allows accessibility of this region to the transcription factor C/EBP (CCAAT/enhancer-binding protein). However, the remodelling of nucleosome is not sufficient for transcription of the IL-12 p40 gene. The IL-12 p40 promoter contains several other elements that are functionally important for its inducible expression (Becker et al., 2001; Ma et al., 1997). A nuclear factor-κb (NF-κB) element was found to be important for promoter activation in response to LPS (Murphy et al., 1995). The c-rel transcription factor has been shown to be a dominant factor to induce IL-12 induction stimulated with LPS in macrophages (Khan et al., 2006; Sanjabi et al., 2000). In the promoter region, a downstream C/EBP site cooperates with rel proteins that bind to the NF-κB site. It was shown that overexpression of C/EBPβ was sufficient for p40 promoter activity (Becker et al., 2001). Although in macrophages isolated from C/EBPβ-deficient mice, transcription of IL-12 p40 gene was enhanced, transcription of IL-12 p35 gene and production of the IL-12 heterodimer were markedly impaired (Gorgoni et al., 2002). An ETS consensus element was found upstream of the NF-κB site might bind to two members of the ETS family of

transcription factors, ETS2 and PU.1, which form a large supramolecular complex with c-rel and several members of the interferon (IFN) regulatory factor (IRF) family, including IRF1, IRF2 and IFN-consensus sequence-binding protein (ICSBP; otherwise known as IRF8) (Salkowski et al., 1999). IRF1 and IRF8 seem to be positive regulatory factors, and IRF2 is a negative regulatory factor, although the expression of p40 is dysregulated in mice that are deficient for any of these three IRFs (Salkowski et al., 1999). Binding of the transcription complex to the ETS site is particularly important for the enhanced transcription of the IL-12 p40 gene when cells were stimulated with IFN-γ and LPS (Ma et al., 1997). Several inhibitors of IL-12 production have been shown to affect one or more of the transcription factors of IL-12. For example, 1,25-dihydroxyvitamin D_3 decreases binding to the NF-κB site, Fc-receptor ligation prevents binding of pu.1 and the transcription complex to the ETS site, and vasoactive intestinal polypeptide affects binding to both sites i.e. NF-κB and ETS sites (D'Ambrosio et al., 1998). A repressor site known as GA12 (GATA sequence in the IL-12 promoter) has been identified between the ETS and NF-κB sites, which is occupied by a GA12-binding protein (GAP12) in unstimulated cells (Becker et al., 2001). Binding of GAP12 was enhanced by treatment with IL-4 and prostaglandin-E_2 (PGE_2), which are two inhibitors of the expression of IL-12 p40. IL-4-mediated early suppression of the IL-12 p40 promoter was shown to be crucially dependent on an intact GA12 sequence (Becker et al., 2001). IL-10 is known to be the most potent inhibitor of transcription of the IL-12 p40 gene. IL-10 is found to inhibit the Inhibitor of NF-κB alpha (IκBα) phosphorylation and degradation preventing nuclear p50/p65 NF-κB and c-rel levels (Rahim et al., 2005). However, the activity of IL-

10 could not be reproduced on a transfected IL-12 p40 promoter (Aste-Amezaga et al., 1998).

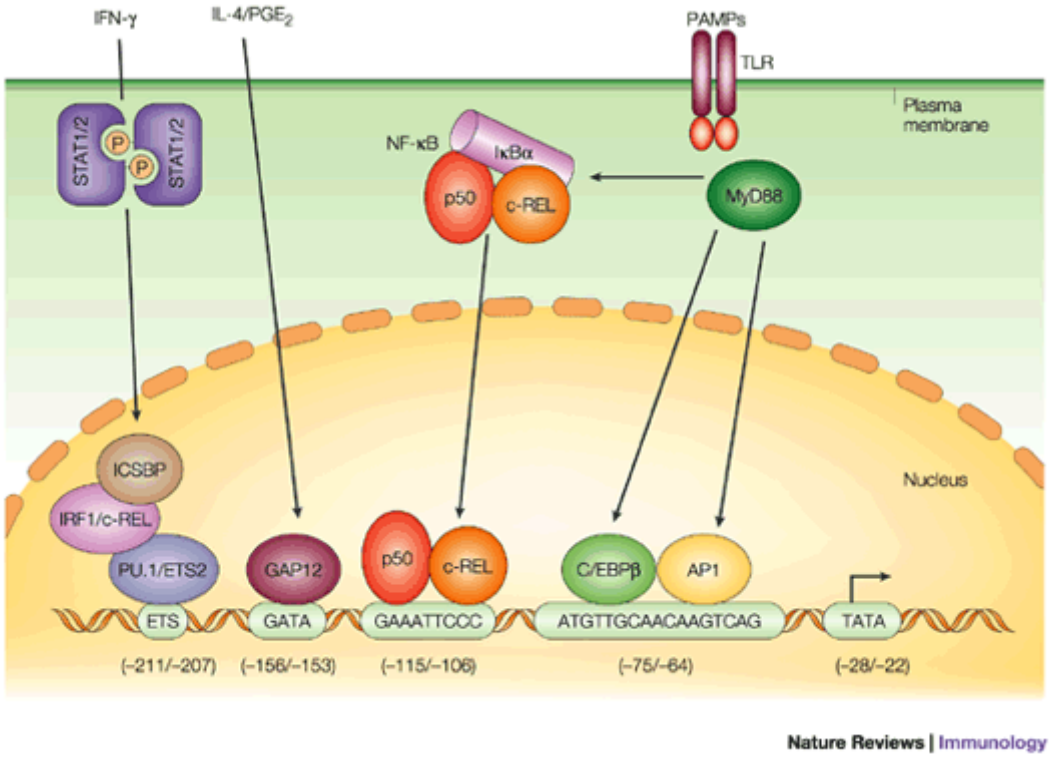

Figure 1.3. Transcriptional regulation of the gene encoding IL-12 p40. As a result of Toll-like receptor (TLR) signalling, nucleosome 1 is selectively remodelled such that it allows accessibility of this region to the transcription factor C/EBP. However, the remodelling of nucleosome 1 is not sufficient for transcription of the gene encoding p40, and the p40 promoter contains several elements that are functionally important for its inducible expression. The NF-κB element was found to be important for promoter activation in response to LPS. A downstream C/EBP site cooperates with c-rel protein that binds to the NF-κB site. An ETS consensus element upstream of the NF-κB site might bind two members of the ETS family of transcription factors, ETS2 and PU.1,which form a large supramolecular complex with c-rel and several members of the IRF family, including IRF1, IRF2 and IFN-consensus sequence-binding protein (ICSBP; otherwise known as IRF8). *(Adapted from Trinchieri G., 2003 Nature Reviews Immunology 3:133-146)*

Analysis of expression of the gene encoding IL-12 p35 was complicated not only by its ubiquitous expression, but also by its low abundance, (Hayes et al., 1995). The promoter regions of both the mouse and the human genes encoding p35 contain putative motifs that bind transcription factors such as SP1, IFN-γ-response element (γ-IRE), PU.1 and C/EBP. The human gene encoding the p35 seems to initiate its transcription from at least two sites, one that is active in B-lymphoblastoid cells and one that is active in monocytes (Hayes et al., 1995; Kadowaki et al., 2001). The latter has a TATA-box-like sequence, which is suggested in the mouse to be a part of an ancestral p35 promoter and generates a shorter mRNA. Multiple transcription start sites for the mouse gene encoding p35 have been identified, which result in four isoforms of p35 mRNA that are present at different levels in unstimulated compared with stimulated cells. Only the mRNA isoforms that accumulate after stimulation readily support translation of the p35 chain, which indicates that expression of p35 is regulated by both transcriptional and translational mechanisms (Babik et al., 1999). The presence of multiple transcription-initiation sites in the human and mouse promoters raises the interesting prospect of differential cell-type usage of the promoter.

1.7. IL-12 and immune response

The major biological activity of IL-12 is on T cells and NK cells in which it increases cytokine production, proliferation, and cytotoxicity. IL-12 is a potent inducer of IFN-γ production from T, NK, and other cell types (Fig. 1.4). Treatment with IL-12 has been

shown to have a marked anti-tumour effect on mouse tumours. IL-12 is found to inhibit establishment of tumours as well as to induce regression of established tumours (Brunda et al., 1993). IL-12 does not induce proliferation of resting peripheral-blood T cells or NK cells, although it augments proliferation of T cells induced by mitogenic lectins, alloantigens, CD3-specific antibodies and phorbol diesters (Perussia et al., 1992), and it has a direct proliferative effect on pre-activated T cells and NK cells.

IL-12 makes the functional bridge between the innate and the adaptive immune response and plays a central role in dictating the T cell effector function viz. helper subset differentiation to Th1 or Th2 in response to infection. IL-12 promotes induction of IFN-γ in T cells and skews the T cell response towards the Th1-type (Hsieh et al., 1993). The role of IL-12 as a Th1 determinant has been analyzed by experiments using recombinant IL-12 *in vitro* and *in vivo*, as well as treating animals with neutralizing antibodies to IL-12. Further, experiments were also carried out by using animals that were genetically deficient for IL-12 p40, IL-12 p35, IL-12Rβ1, IL-12Rβ2 or STAT4 (Trinchieri, 1998). The IFN-γ activated phagoytic cells are also primed to produce higher levels of IL-12 in a powerful positive feedback mechanism (Kubin et al., 1994). Although IFN-γ is not an absolute requirement for optimal IL-12 production, this feedback mechanism is particularly powerful in regulating IL-12 levels mostly at the transcriptional level of both p40 and p35 IL-12 genes. At least two sites on the p40 promoter, an ETS-2 and NF-κB element have been shown to be required for transcriptional activation of IL-12 p40 and responsiveness to IFN-γ stimulation and

development of autoimmune inflammation. The ability of IL-12 to induce, maintain and amplify antigen-specific Th1 responses is essential to control infections with microbial pathogens (Trinchieri, 1998). The Th1 T cells are characterized by expression of T-bet, a member of the T-box family of transcription factors that acts in part by inducing IFN-γ (Szabo et al., 2000). So the finding that IL-12 is either unable or poorly efficient in inducing the expression of the transcription factor T-bet, has led to the hypothesis that IL-12 is required more for the expansion and optimal activation of Th1 T cells rather than for the initiation of the Th1 response (Mullen et al., 2001).

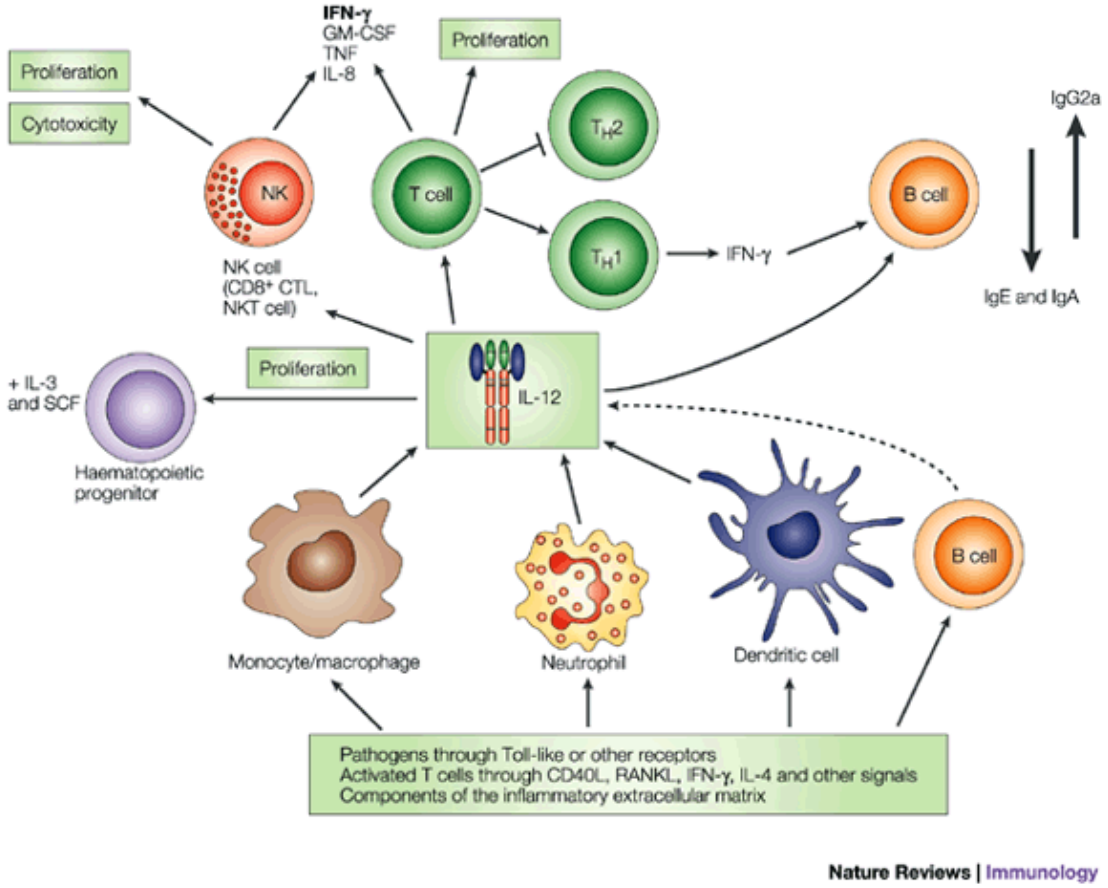

Figure 1.4. The diagram showing activities of IL-12 produced by different cell types. The IL-12 is mainly produced by the monocytes/macrophages, neutrophils and dendritic cells in response to pathogen stimuli like bacteria, fungi, parasites and viruses through TLRs and other receptors. IL-12 favors IFN-γ production, induction of Th1 T cells and activates NK cells cytotoxic activity. The Th2 T cell response is inhibited by IL-12. *(Adapted from Trinchieri G., 2003 Nature Reviews Immunology 3:133-146)*

1.8. The role of calmodulin in the regulation of IL-12

Calmodulin (CaM) is a Ca^{2+}-sensor protein that converts Ca^{2+} signals into cellular function. CaM is expressed in all eukaryotic cells where it participates in signaling pathways that regulate many crucial processes such as growth, proliferation and movement. It is relatively small (vertebrate CaM has 148 residues), evolutionarily highly conserved and comprises four E–F hands. The first two E–F hands combine to form a globular N-terminal domain that is separated by a short flexible linker from a highly homologous C-terminal domain consisting of E–F hands 3 and 4 (Chin and Means, 2000). CaM has high affinity for Ca^{2+} (K_d = 5 × 10^{-7} M to 5 × 10^{-6} M), that falls within the range of intracellular Ca^{2+} concentrations exhibited by most cells (10^{-7} M to 10^{-6} M). CaM adopts two different conformations depending on the absence or presence of Ca^{2+} (Fig. 1.5). The N-terminal domain of the apo-CaM molecule adopts a 'closed' conformation in the absence of Ca^{2+} and both E–F hands are packed together whereas the C-terminal domain of apo-CaM adopts a 'semiopen' conformation in which a partially exposed hydrophobic patch is accessible to solvent. This might allow the C-terminal domain of CaM to interact with some target proteins at resting levels of intracellular free Ca^{2+} (Swindells and Ikura, 1996). In contrast, if there is rise in Ca^{2+}, the Ca^{2+} ion is coordinated in each Ca^{2+}–binding loop of Ca^{2+}–CaM by seven carboxylate, ligands. The binding of Ca^{2+} leads to substantial alterations in the inter-helical angles within the E–F hands in each domain and dramatically changes the two domains of CaM to produce more 'open' conformations (Fig. 1.5)

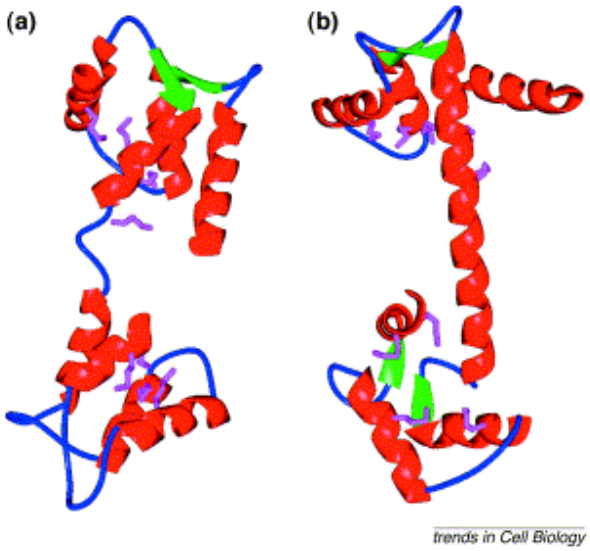

Figure 1.5. The Ca^{2+}-regulated conformational change in Calmodulin The main chain structure of Ca^{2+}-free (apo) CaM (a) and $Ca^{2+}{}_4$-CaM (b) are shown in red with their respective N-terminal domain on top. Methionine side chains are shown in purple to denote the location of potentially hydrophobic pocket in each of the two domains. Ca^{2+}binding produces large change in the helices in both domain, resulting in the exposure of several hydrophobic residue. *(Adapted from David Chin and Anthony R Means, Trends in Cell Biology, Volume 10, Issue 8, 322-328, 1 August)*

CaM constitutes about 0.1% of the total protein present in cells (10^{-6} M – 10^{-5} M) and is expressed at even higher levels in rapidly growing cells. The local intracellular availability of CaM is biologically significant because functions of many proteins are regulated by the CaM protein. It has been reported that two members of the NF-κB/rel family, the c-rel and the p65 interact directly with the Ca^{2+}-CaM protein (Antonsson et al., 2003). The c-rel and the p65 interact with CaM through a similar sequence near the nuclear localization signal (Antonsson et al., 2003). CaM binds to both c-rel and p65 after their release from IκBα but it can specifically inhibit nuclear import of c-rel while letting p65 translocate to the nucleus and acts on its target genes. CaM can therefore, differentially regulate the activation of NF-κB/rel proteins following stimulation (Antonsson et al., 2003). It has been observed that H_2O_2 inhibits IL-12 induction in activated macrophages by targeting the CaM protein in the cytosol. The CaM inhibitor trifluoperazine increased both nuclear c-rel and IL-12 p40 levels in H_2O_2-treated macrophages (Khan et al., 2006), emphasizing a critical role of CaM in the regulation of IL-12 p40 during oxidative stress.

1.9. Effects of ROS and antioxidant in immune functions

ROS encompass a wide range of molecules including superoxide (O_2^-) and other oxygen derived intermediates such as H_2O_2, hydroxyl radical ($^{\cdot}OH$) etc that play an important role in host resistance to microbial pathogens. Decades of research have provided a detailed understanding of the regulation, generation and actions of these mediators, as well as their roles in resisting infection. However, differences of opinion remain with

regard to their host specificity, cell biology, sources and interactions with one another (Fang, 2004).

ROS are mainly generated by either a respiratory burst of polymorphonuclear granulocytes through reduced NADPH phagocyte oxidase system or mitochondrial oxidative metabolism. The NADPH oxidase system, comprises of membrane bound $gp91^{phox}$ (where *phox* stands for phagocyte oxidase), $p22^{phox}$, a small G-protein Rap1A and cytosolic $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, small G-proteins Rac2 and Cdc42. The membrane-bound subunits $gp91^{phox}$ and $p22^{phox}$ together form the heterodimeric cytochrome b_{558}. Upon oxidase activation, the cytosolic subunits translocate to the plasma membrane and bind with the cytochrome b_{558} complex. Additionally, the small GTPase proteins Rac2, Cdc42, and Rap1A are involved in the assembly and activation of the NADPH oxidase. A new protein, p29 peroxiredoxin that is associated with the oxidase proteins (mainly $p67^{phox}$) has recently been described (Sheppard et al., 2005).

Increased levels of ROS in cells can result in oxidative stress and cause cellular damage. Indeed, such damage is associated with the initiation and progression of many diseases, including neurodegenerative disorders, diabetes, atherosclerosis, and cancer. ROS are involved as a key second messengers in numerous signaling pathways, eliciting a diverse array of biological responses ranging from transcriptional regulation, differentiation and proliferation (Martindale and Holbrook, 2002; Storz, 2006). ROS have been implicated to have a critical role in immune response (Rahman,et al. 2006 aand Yodoi, et al 1992).

ROS are known important regulators of transcription factors controlling macrophage effector and APC functions (Gloire et al., 2006)**.** The free radicals act to modulate the metabolism of arachidonic acid and prostaglandins. Neutrophil and macrophage phagocytosis stimulates the respiratory burst important for killing various pathogens (Knight, 2000).

Free radical scavengers or antioxidants were found to block the mesangial VCAM-1 and NF-κB proteins, implying that oxidative stress provides an important regulatory signal in the pathogenesis of glomerular mesangial cell disorders (Khachigian et al., 1997). The role of ROS in cytokine mediated degradation of sphingomyelin to ceramide leading to apoptosis has been underscored (Singh et al., 1998). This study emphasizes unique role of ROS in the regulation of cytokine-mediated cytotxicity leading to apoptosis in human brain cells in patients with neuro-inflammatory diseases like multiple sclerosis and X-adrenoleukodystrophy. Various studies indicate that an imbalance in ROS generation can contribute to an increased susceptibility to cancer and infections in severe humoral immunodeficiency disease (Reichenbach et al., 2000). Oxidative stress thus has an immense influence on the immune responses of the host. Oxidative stress influences the acquired immune response by activation of NF-κB which regulates transcription of genes of cytokines, chemokines and cell adhesion molecules (Morel and Barouki, 1999). In mammals, the NF-κB family consists of NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-rel and RelB, all characterized by the presence of the rel homology domain (RHD), which mediates DNA binding, dimerization between members of this family and

association of NF-κB dimers with regulatory proteins of the IκB group (Hayden and Ghosh, 2004). Normally, NF-κB complexes lie latent in the cytosol, bound to IκBs. A wide range of stimuli are capable of activating NF-κB from these inactive cytosolic pools, a process that typically involves sequential phosphorylation and proteolytic degradation of IκB inhibitors by the IκB kinase (IKK) complex (consisting of the subunits IKKα, IKKβ and NEMO) and the ubiquitin/proteasome system, respectively (Hayden and Ghosh, 2004). Treatment of cells with antioxidants such as *N*-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) blocked activation of NF-κB by an array of stimuli, including TNF-α, IL-1β, phorbol 12-myristate 13-acetate (PMA), LPS, double-stranded (ds)RNA, cycloheximide and H_2O_2, (Gloire et al., 2006; Schreck et al., 1991). The induction of NF-κB was also abrogated by the overexpression of ROS-scavenging enzymes such as catalase, GSH-PX, thioredoxin peroxidase and Mn^{2+} superoxide dismutase (Mn-SOD) (Bowie and O'Neill, 2000; Gloire et al., 2006). ROS play important roles in signaling leading to the activation of programmed cell death (Matsuzawa et al., 2005).

1.10. The roles of mitogen-activated protein kinases (MAPKs) in the regulation of IL-12

The function and regulation of MAPKs have been conserved during evolution from unicellular organisms to multi-cellular organisms (Widmann et al., 1999). Many extracellular stimuli elicit specific biological responses through the activation of MAPK

cascades. Multicellular organisms have three well-characterized subfamilies of MAPKs that control a vast array of physiological processes. These MAPKs include the extracellular signal-regulated kinases (ERK1 and ERK2), the c-Jun NH_2-terminal kinases (JNK 1, JNK 2, and JNK 3) and the four p38 MAPKs. MAPKs catalyze phosphorylation of substrate proteins that include other protein kinases, phospholipases, transcription factors, and cytoskeletal proteins (Johnson and Lapadat, 2002). It has been clearly established that LPS activates all three MAP kinases in monocytes (Liu et al., 1994). However, the relationship between the activation of these kinases and the induced cytokine expression remains obscure.

Four p38 MAP kinases are well characterized that include α, β, γ and δ. The p38-α MAPK is the best characterized and is expressed in most cell types. The p38 kinases were first defined in a screen for drugs inhibiting TNF-α-mediated inflammatory responses (Lee et al., 1994). The p38 MAPKs regulate expression of many cytokines including the IL-12. Although many studies indicate that the p38 MAPK promotes IL-12 p40 induction (Kim et al., 2005), several studies have shown that a pharmacological inhibitor of p38 MAPK, SB203580 can upregulate IL-12 in LPS/LPS+IFN-γ-activated macrophages (Kim et al., 2004; Marriott et al., 2001; Salmon et al., 2001; Xie et al., 2003) indicating that p38 MAPK may have opposing roles in IL-12 induction (Salmon et al., 2001; van den Blink et al., 2001). Thus, probably the p38 MAPK activity during macrophage stimulation is critical in regulating IL-12 in a positive or negative fashion. The negative regulation of p38 MAPK involves the CAM-c-rel signaling (Boddupalli et al., 2007). A direct regulation

of p38 MAPK on the nuclear c-rel probably determines IL-12 p40 transcription to be positive or negative. Activated p38 MAPK can positively influence IL-12 p40 transcription, but the p38 MAPK activity above a critical level, upregulates CAM level and hence can prevent nuclear translocation of c-rel and consequently IL-12 p40. The p38 MAPKs are also activated by many other stimuli, including hormones, ligands for G protein-coupled receptors, and stresses such as osmotic shock and heat shock.

The relationship between MAP kinase activity and cytokine induction in human monocytes is not very clear. The c-Jun N-terminal kinase (JNK) plays an important role in cytokine expression. Studies by Utsugi, et al. (2003) have shown that JNK negatively regulates lipopolysaccharide-Induced IL-12 productions in human macrophage as inhibition of JNK activation by SP600125 dose-dependently enhances both LPS-induced IL-12 p40 production from THP-1 cells and IL-12 p70 production from human monocytes. Furthermore, JNK antisense oligonucleotides attenuated cellular levels of JNK protein and LPS-induced JNK activation, but augmented IL-12 p40 protein production and mRNA expression (Utsugi et al., 2003).

1.11. Tuberculosis: modulation of immune system and immunoadjuvant

Tuberculosis, caused by *Mycobacterium tuberculosis* (*Mtb*), is one of the main factors of human morbidity and mortality. In recent years there has been a significant increase in the incidence of tuberculosis because of the emergence of multi-drug resistant strains

and increased numbers of highly susceptible immuno-compromised individuals arising from the AIDS pandemic. About 2 million people die with 9.2 million new cases, mostly in the developing countries; arise of tuberculosis disease each year (WHO report, 2004).

Tuberculosis is primarily a respiratory disease, but also affects other body parts like bone, lymph nodes, circulatory and nervous system. Tuberculosis infection is initiated after inhalation of viable bacilli present in exhaled droplets by a person with active infection. Once in the lung, the bacilli are phagocytosed by alveolar macrophages. Inside the macrophage, *Mtb* modulates the activity of the phagosome by preventing its fusion with acidic, hydrolytically active lysosomes (Sturgill-Koszycki et al., 1994). In experimental hosts, this marks the start of a period of 'rapid' division in which the bacteria grow exponentially until the emergence of an acquired immune response. Internalization of the bacilli also triggers a proinflammatory response that induces the macrophages to invade the nearby epithelium. This response leads to the recruitment of mononuclear cells from neighboring blood vessels. These monocytes form the cellular matrix of the early granuloma, which is the primary characteristic of this disease. In its early stage, the granuloma has a core of infected macrophages enclosed by foamy macrophages and other mononuclear phagocytes, surrounded by lymphocytes. As the granuloma matures, it develops an extensive fibrous capsule that surrounds the macrophage core and excludes the majority of lymphocytes from the center of the structure. Concomitant with this transition is a considerable decrease in the number of blood vessels penetrating the granuloma. At this stage there is a noticeable increase in

the number of foamy macrophages in the fibrous capsule. These cells are responsible for the accumulation of caseous debris in the center of the granuloma, which marks the progression to active disease. In an immunocompetent person, this progression is localized to individual granulomas, and the same tissue site contains other granulomas that seem to be under perfect immune containment. Nonetheless, in a progressive infection, the caseous, necrotic center of the granuloma liquefies and cavitates, spilling thousands of infectious *Mtb* into the airways. This damage to the lungs triggers the development of a productive cough, which facilitates generation of the infectious aerosol and completion of the bacterium's life cycle (Fig. 1.6) (Russell et al., 2009).

In animals, and in humans, there is a phase of blood-borne spread ~3 weeks after unimmunized individuals are first infected. In 90–95% of individuals, the infection remains latent. The molecular factors or environmental conditions that influence the progression of latent phase to active disease are not very clear. Recent studies have indicated that pathogen-induced dysregulation of host lipid synthesis and sequestration serves a critical role in this transition (Russell et al., 2009). Progressive disease is characterized by weight loss, toxicity of TNF-α, cavitation and fibrosis, even though IFN-γ produced by Th1 cells can decrease the amount of fibrosis (Rook et al., 2005).

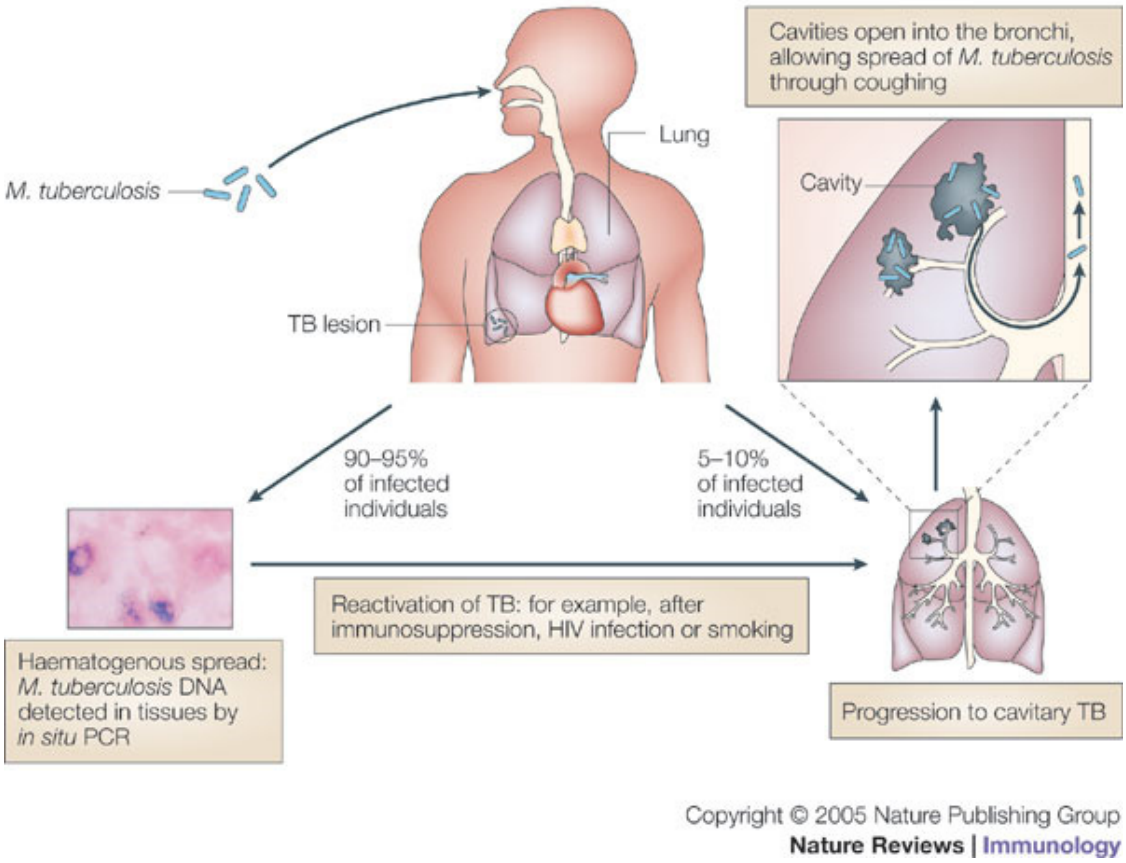

Figure 1.6. Tuberculosis (TB) is spread by aerosols and can establish infection with or without a visible primary lesion *(Adapted from Praham A.W. Rook et. al., Nature Review Immunology 5, 661-667 (August 2005*)

Tuberculosis has been declared a global emergency by the World Health Organization (WHO report 2004). A number of conditions that are associated with altered host cellular immunity like HIV infection, extremes of age, immunosuppressive therapy, cancer, end stage renal disease, diabetes, severe malnutrition, etc. do increase the risk of developing active tuberculosis. *M. tuberculosis* is inextricably intertwined with humankind in terms of both physiology and social development and has been in the spotlight of intense international research for the past several decades. The ponderous causal agent *M. tuberculosis* is considered to be one of the most successful predator of the mankind and has remained unconquered despite tremendous medical and technical advances. At least three drugs need to be administered continuously for 6 months to eradicate TB from an infected person. No new drugs have been added to the first-line treatment regimen for TB for more than 30 yrs.

Mycobacterium bovis bacilli Calmette–Guerin (BCG) is the only vaccine currently available against TB. BCG is an attenuated strain of *M. bovis* that was derived from a virulent strain at the start of the last century, after more than 13 years of continuous *in vitro* passage. The current BCG vaccine protects against severe childhood forms of disease, including pulmonary and extrapulmonary TB and the often fatal TB meningitis. It also confers protection against leprosy. The WHO recommends BCG vaccination in areas of high TB occurrence. It is a very safe vaccine with good adjuvant activity. It elicits both humoral and cell-mediated immune responses. It can be provided at birth or any time thereafter and a single dose can produce long-lasting immunity. However, the level

of protection conferred by BCG is extremely variable; it differs according to the form of pulmonary TB. For example one study shows that BCG provides no protection in Malawi but 50%–80% protection in the United Kingdom (Black et al., 2002). The reasons for the failure of BCG have been widely debated. Present hypotheses for the failure of BCG in developing countries include:

- The rapid destruction of BCG by Th1 T cell responses that are elicited by cross-reactive mycobacteria
- Phenotypic changes in the vaccine during passage from the original cultures and in the manufacturing process
- The variations in the strains of BCG that are used and the strains of *M. tuberculosis* that are encountered
- The presence of Th2 T cell responses, which are driven by co-existing helminth infections and
- Variability in dose, route of administration, age of administration and genetic differences among vaccines.

Given the variable protective efficacy generated by BCG vaccine against TB, there is a concerted effort worldwide to develop approaches to improve the efficacy of the existing BCG vaccines that could be used to reduce the burden of TB. Resistance to *M. tuberculosis* involves activation of *M. tuberculosis* specific CD4+ and CD8+ T cells by APCs in host. One of the factors important for activation of Th1 T cells is IL-12 secreted by APCs. IL-12 increases the production of IFN-γ and induces cell-mediated immune

response by increasing macrophage activation, NK cell activity and Th1 T cell response - all are crucial for *Mtb* immunity (Russell et al., 2009). It has been observed that individuals deficient in receptors for IFN-γ and/or IL-12 are extremely susceptible to mycobacterial infections. These data suggest crucial requirements of IFN-γ/IL-12 for activation of protective immunity in the host (Flynn and Chan, 2001). Studies in animal models indicate that the most effective vaccine strategy should be those that stimulate T cell responses, both CD4+ and CD8+, to produce Th1-associated immune response.

The recombinant BCG (rBCG) techniques may be useful for the development of a more effective mycobacterial vaccine than the parental BCG now in use. Various strategies have been used to develop rBCG against mycobacteral diseases. One is based on rBCG producing large amounts of autologous protective antigens; these supplementary antigens are designed to enhance immunity to other BCG antigens by increasing the expression of their genes, as is the case for the immunodominant TB antigens. In one study, a recombinant BCG over-expressing Ag85B-Rv3425 (rBCG::AG85B-Rv3425) fusion protein was developed (Qie et al., 2008). It was observed that the level of antigen-stimulated T cells expressing IFN-γ was significantly higher in the C57BL/6 mice vaccinated with rBCG::Ag85B-Rv3425 than with BCG alone. In a separate study, the recombinant BCG vaccine expressing and secreting the 30-kDa, Ag85A was found to provide better host survival after challenge than the parental BCG in a guinea pig model of pulmonary TB. Animals immunized with rBCG30 when challenged with a virulent

strain of *Mtb* survived significantly longer than the control animals immunized with BCG only (Horwitz and Harth, 2003).

Alternatively, BCG genes that have been lost by deletion from parental *M. bovis* strain can be extremely important for eliciting effective immune response when incorporated in the BCG strain. An example is the case of ESAT-6 deleted from region RD1 of BCG. In a study, recombinant BCG overexpressing complete RD-1 (BCG::RD-2F9), that secrete ESAT-6 was generated and used to vaccinate mice and guinea pigs. These immunized animals were better protected against challenge infection with *M. tuberculosis*, with less severe pathology and reduced dissemination of the pathogen, as compared with the control animals immunized with BCG alone (Pym et al., 2003).

There are also attempt to use cytokines to enhance the efficacy of BCG vaccine. Murray et al. (1996) constructed recombinant BCG (rBCG) strains that were able to secrete cytokines such as IL-2, granulocyte-macrophage colony-stimulating factor (GMCSF), and IFN-γ. Antigen specific proliferation and cytokine release were found to be substantially greater with splenocytes derived from mice injected with rBCG strain secreting IL-12/GMCSF/IL-2 cytokines than with splenocytes from mice injected with BCG lacking the cytokine genes (Murray et al., 1996). The IL-12 therapy alone can improve protection against *Mtb* chaqllange infection in BALB/c mice (Flynn et al., 1995).

Formulations that induce the production of Th1 responses also are desirable and doubtless an essential element of a successful vaccine. One of the major difficulties in developing an effective BCG vaccine is the need of effective adjuvants available for clinical trials. The paucity of adjuvants is reflected by the fact that the only adjuvant licensed for world-wide usage is an aluminium compound identified more than 70 years ago as it has immunostimulatory properties. In addition, the oil-in-water formulation designated MF59 has received licensure in some countries as part of an influenza vaccine along with virosomes used in both influenza and hepatitis A vaccines (O'Hagan et al., 2007). But both of these adjuvants are characterized by inducing humoral immune responses and are thus effective in elevating serum antibody titers. However, their ability to elicit cell-mediated immune responses (CMI) that is essential for anti-tuberculosis immunity is limited. Some new adjuvants or immunomodulators capable of inducing potent T cell responses have been thus studied. Recently, one group used a liposomal adjuvant designated cationic adjuvant formulation (CAF01) to improve efficiency of BCG. This adjuvant is based on liposomes formed by N,N′-dimethyl-N,N′-dioctadecylammonium (DDA) with the synthetic mycobacterial immunomodulator α,α′-trehalose 6,6′-dibeheneate (TDB) inserted into the lipid bilayers (Agger et al., 2008). It was demonstrated that compared to commercially available adjuvants, CAF01 was particularly effective in generating strong cellular immune responses and in addition a strong antibody response as shown by high titers of IgG2a. CAF01 was found to give rise significant levels of protection against challenge infection to *M. tuberculosis.* In another study interleukin-12 (IL-12) and oligodeoxynucleotides (ODN) containing cytidine

phosphate guanosine (CpG) motifs were chosen as adjuvants to increase the effectiveness of BCG vaccine. Both IL-12 and ODN containing CpG motifs were examined for their ability to enhance the efficacy of BCG. It was observed that the bacterial loads in mice vaccinated with BCG plus IL-12 or CpG ODN were two- to five fold lower than those of mice vaccinated with BCG alone (Freidag et al., 2000).

1.12. Aims and Objectives

The redox status of the cell can regulate the innate immunity presumably by acting as signaling regulator that can control a number of immunoregulatory genes. The intracellular redox environment is mainly controlled by the glutathione-redox which is defined as the ratio of GSH to GSSG (Filomeni et al., 2005) and plays critical roles in maintaining cellular homeostasis and various physiological functions (Rahman and Adcock, 2006). The cellular glutathione-redox dynamically regulates protein functions by reversible disulfide bond formation in a variety of proteins including phosphatases, kinases and transcription factors (Biswas et al., 2006; Dominici et al., 1999; Rahman et al., 2005) and is known to protect cells against oxidative stress (Qanungo et al., 2007; Reynaert et al., 2006). The glutathione-redox balance in macrophages is shown to be critical for mounting innate immune responses and has been implicated in several pathophysiological conditions (Koike et al., 2007; Nakamura et al., 1997; Rahman and Adcock, 2006; Yodoi and Uchiyama, 1992), indicating that the glutathione-redox balance can be targeted for designing immunotherapeutics to control these conditions. However redox-based therapy is still in its early developmental phases.

It has been observed that triggering of macrophage receptors by pathogen products could change the macrophage redox status (Nakamura et al., 1997) and modulate the cytokine milieu (Akira et al., 2006; Rakoff-Nahoum et al., 2004). Various studies indicate that glutathione levels in antigen presenting cells can influence the T cell response

(Murata et al., 2002; Peterson et al., 1998). It is well known that the Th1/Th2 T cell lineage commitment depends to a great extent on the cytokine milieu induced during the innate phase of activation of macrophages (Banchereau and Steinman, 1998; Reinhardt et al., 2006). For example, IL-12 and TNF-α activate the Th1 T cell whereas IL-10 cytokine favors the Th2 T cell development. The IL-12 cytokine increases production of IFN-γ and stimulates cell-mediated immunity. The Th1 immunity is crucial for conferring protection against various intracellular pathogens including *M. tuberculosis*. Therefore, over the past few years, there have been attempts to use IL-12 as an immunoadjuvant in tuberculosis (TB) vaccines (Gurunathan et al., 1998; Holland, 2001). The TNF-α also plays an important role for granuloma formation and induction of cytotoxicity against the bacilli (Roach et al., 2002; Senaldi et al., 1996). Therefore, understanding how various macrophage effector-APC functions are modulated by the intracellular glutathione-redox balance is likely to provide insights on how the T cell micro-environment is modulated during various pathophysiological disorders and will be helpful to design novel immunotherapeutics to treat these disorders. However, the signaling events by which glutathione-redox can directly control macrophage innate functions and cytokine production are largely not understood, other than few cases like GSH-mediated regulation of NF-κB (Qanungo et al., 2007; Reynaert et al., 2006).

TB is known to be a killer disease with impressive credentials. Over the past 2 centuries, it has killed more than a billion people. Currently tuberculosis is the leading cause of mortality from a single infectious agent accounting for 26% of all preventable adult

deaths in the developing world (Bloom and Murray, 1992) and in the coming decade, it is slated to kill at least 30 million people unless efforts to control its transmission and deliver effective treatment in a timely fashion are made. The tuberculosis crisis is further aggravated by the emergence of multidrug-resistant (MDR) bacilli. Standard anti-microbial treatments are complicated and associated with frequent and possibly severe side effects and sub-optimal treatments with anti-tuberculosis drugs are considered to be one of the important factors for emergence of the MDR bacilli. The current BCG vaccine is controversial for its efficacy to control the TB epidemic. In such situation, alternative approaches such as adjuvant immunotherapy appears to be promising for the management of TB since it offers several potential advantages over chemotherapy. Evidence indicates that Th1 immune response is supressed in patients with active TB infection (Baliko et al., 1998). Interestingly, studies also indicate that TB patients have altered glutathione balance (Venketaraman et al., 2008). However, it is not clear whether anti-TB Th1 response can be boosted by modulating macrophage effector-APC functions by altering the glutathione-redox balance of the macrophages. Therefore, in the present study,

1) The ability of the glutathione-redox to modulate macrophage innate-effector functions like cytokine and NO production were investigated.

2) The innate signaling pathways modulated in macrophages by the intracellular glutathione-redox affecting these macrophage functions were also determined and finally,

3) The possibility of glutathione to be used as an immunotherapeutic adjuvant in tuberculosis was investigated.

Chapter 2 - Materials and Methods

2.1. Animals

The BALB/c mice were bred and maintained in the animal facility of Indian Immunological Limited (IIL), India. All the mice were 6-12 weeks old and experiments were performed as per the guidelines laid by the Institutional Animal Care and Usage Committee of IIL.

2.2. Collection of peritoneal exudate cells (PECs)

The PECs were harvested by injecting 4% thioglycolate broth. BALB/c mice of both sexes were used at 3-6 months of age. Murine peritoneal macrophages were elicited by intraperitoneal injection of 1.0 ml of thioglycollate per mouse. Mice were killed by CO_2 asphyxiation 3 days later, and peritoneal exudate cells were harvested by peritoneal cavity lavage with chilled sterile tissue culture medium.

2.3. Isolation of monocyte-derived macrophages (MDM)

MDM were purified following the method as described earlier (Gan et al., 1993). Briefly, the Peripheral Blood Mononuclear Cells (PBMCs) were isolated from healthy volunteers by Ficoll-Hypaque (Sigma-Aldrich, St Louis, MO) density gradient centrifugation. Cells were washed and cultured in RPMI-1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and antibiotics

(Invitrogen) (RPMI-10). Macrophages were generated by culturing adherent monocytes in RPMI-10 supplemented with 0.1 ng/ml GM-CSF, (Sigma-Aldrich) at 37°C and 5% CO_2 for a week. Medium was replaced with fresh RPMI-10 containing 0.1 ng/ml Granulocyte-macrophage colony-stimulating factor (GM-CSF) on alternate days.

2.4. Macrophage stimulation assay

The RAW 264.7 macrophages were obtained from NCCS (National Centre for Cell Science, Pune, India) and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) containing 10% FBS and antibiotics (DMEM-10). MDM or RAW 264.7 macrophages were plated at a density of 3 x 10^6 cells/ml and treated with various concentrations of N-acetyl-L-cysteine (NAC) (Sigma-Aldrich) or glutathione ethyl ester reduced (GSH-OEt) (Sigma-Aldrich). Wherever needed, L-buthionine-(S,R)-sulphoximine (BSO, Sigma-Aldrich), SB203580 (Sigma-Aldrich), trifluoperazine (TFP, Sigma-Aldrich) were added 30 min prior to treatment with NAC/GSH-OEt. Culture supernatants were harvested after 48 h to estimate levels of IL-12 p40 and IL-12 p70, IL-10 and TNF-α cytokines by two site sandwich enzyme immune assay (EIA) (BD Biosciences, Pharmingen, San Diego, CA) as described earlier (Khan et al., 2006; Rahim et al., 2005). The p50 NF-κB, p65 NF-κB, c-rel transcription factors, IκBα, calmodulin protein, p38 Mitogen-activated protein kinase (MAPK) and suppressor of cytokine signaling 1 (SOCS1) levels were measured after 45-60 min of NAC treatment or at indicated time points by

either immunoblotting or EIA or Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or flow cytometry or immunofluorescence microscopy.

2.5. Cytokine assay

The IFN-γ, IL-5, IL-10, IL-12 p40/70 and TNF-α cytokines in the macrophage culture supernatants were quantified by two-site sandwich EIA (BD Biosciences Pharmingen). In brief, the 96-well polyvinyl chloride microtiter plates were coated with respective purified capture antibody (Ab) at 1:250 dilutions in coating buffer (0.1 M carbonate buffer, pH 9.5) and were incubated for overnight at 4°C. The plates were washed with wash buffer (PBS with 0.05% Tween-20) and blocked with 10% FBS in PBS followed by incubation with the test samples for overnight at 4°C. After washing, plates were incubated with biotin conjugated detection Ab against the respective capture Ab followed by incubation with streptavidin-horseradish peroxidase (HRP). The HRP activity was detected using a chromogenic substance o-phenylenediamine tetrahydrochloride (Sigma-Aldrich) at 0.5 mg/ml in citrate-phosphate buffer (pH, 5.4) containing 1 µl/ml hydrogen peroxide (H_2O_2, Qualigen, Mumbai, India). The reaction was terminated using 1 N H_2SO_4, and the absorbance value was measured at 492 nm. Standard curve for the cytokine was obtained using the recombinant standard protein provided in the kit.

2.6. Immunofluorescence assay for IL-12 p40 and SOCS1

Immunofluorescence assay for IL-12 p40 was carried out as described earlier (Rahim et al., 2005). For IL-12 p40 immunofluorescence assay, briefly, at 2 h post treatment with NAC (20 mM), cells were incubated with brefeldin A (Sigma-Aldrich) at 20 µg/ml for 4 h, washed and fixed with 3% paraformaldehyde (Sigma-Aldrich) for 30 min. Cells were then permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 15 min. Cells were blocked with 2% BSA (Sigma-Aldrich) and incubated with rat anti-IL-12 p40 Ab (BD Biosciences Pharmingen) followed by anti-rat Fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich). For checking SOCS1 expression, RAW 264.7 macrophages were treated with various concentrations of NAC. After 1 h, cells were fixed and permeabilized and incubated with goat anti-SOCS1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by incubation with anti-goat FITC (Sigma-Aldrich). Cells were washed and embedded in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Microscopy was performed on a Nikon fluorescence microscope (Nikon DX1, Tokyo, Japan).

2.7. Total RNA isolation and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) for IL-12 p40 and SOCS1

For RT-PCR, RAW 264.7 macrophages were treated with varying doses of NAC. Total RNA was extracted from various experimental groups using Trizol (Invitrogen). Briefly, the cells were lysed by adding 1 ml of trizol reagent followed by incubation for 5 min at room temperature. To the lysate, 0.2 ml chloroform per 1 ml of trizol was added, mixed by vortexing for 15 sec and the whole mixture was centrifuged at 14,000 rpm for 15 min at 4°C. Following centrifugation, the mixture got separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was transferred into a new tube and the RNA from the aqueous phase was precipitated by adding 0.5 ml isopropanol, centrifuged at 14,000 rpm for 10 min at 4°C. The RNA pellet was washed with 500 µl of 75% ethanol, centrifuged at 14,000 rpm for 2 min at 4°C and air dried. Finally the pellet was dissolved in RNase-free water. Reverse transcription was carried out using 2 µg of total RNA using Moloney Murine Leukemia Virus reverse transcriptase (Invitrogen). The PCR was performed at an annealing temperature of 58°C with IL-12 p40 forward primer (5'-ATGTGTCCTCAGAAGCTAAC-3') and reverse primer (5'-TCCTAGGATCGGACCCTG-3'), which generated a product of 1010 bp (Jia et al., 2003). For SOCS1, PCR was performed at an annealing temperature of 46°C using forward primer 5'-CACCTTCTTGGTGCGCGACA-3') and reverse primer (5'-GCAGCTCGAAAAGGCAGTCG-3') (Shoda et al., 2007) which generated a product of 150 bp. The house keeping gene β-actin was used as an internal control and PCR was

performed using the forward primer (5'-GTGGGCCGCTCTAGGCACCA-3') and reverse primer (5'-CGGTTGGCCTTAGGGTTCAGGGGGG-3'), which generated an amplicon of 244 bp (Khan et al., 2007). The amplified products of IL-12 p40, SOCS1 and β-actin were resolved by electrophoresis on 1.2% agarose gels and visualized by ethidium bromide staining.

2.8. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

RAW 264.7 macrophages were either left untreated or treated with various concentrations of NAC and cultured as 3 x 10^5 cells/well in 96-well tissue culture plates in 200 µl volume of medium. After 48 h, MTT (Sigma-Aldrich) was added as 1 mg/ml and incubated further for 4 h (Khan et al., 2006). Cells were lysed overnight using 100 µl of lysis buffer. The absorbance was determined at 570 nm.

2.9. Immunoblot analysis

To detect the levels of p50, p65 and c-rel transcription factors, cytoplasmic and nuclear extract were prepared. In brief, cells were harvested from each experimental group, centrifuged and the pellets were washed with ice cold PBS. To the washed pellet 250 µl of ice-cold cytoplasmic extraction buffer (30 mM Tris, 10 mM Magnesium acetate, 1% NP-40, 1 mM Sodium orthovanadate) containing cocktail of freshly prepared protease

inhibitors (2 μg/ml Leupeptin, 3 μg/ml Aprotenin and PMSF) were added. The cell suspension was incubated on ice and vortexed vigorously for 2-3 min for the rupture of the plasma membrane. The contents were centrifuged for 1 min at 10000 rpm at 4°C and supernatants (cytoplasmic extracts) were transferred to a pre-chilled microfuge tube and dialyzed in PBS. The nuclear pellets were again washed with cytoplasmic extraction buffer and recentrifuged to remove any residual cytoplasmic material. Fifty µl of ice-cold nuclear extraction buffer (10 mM HEPES, 25% glycerol, 142 mM NaCl, 0.5 M EDTA, 0.1 M DTT and cocktail of protease inhibitor) was added to the pellet containing nuclei. This was incubated on ice for 45 min with intermittent vortexing and then centrifuged for 20 min at 14000 rpm at 4°C. The supernatants (nuclear extracts) were collected and dialyzed in PBS. The dialyzed preparations of the cytoplasmic extracts and nuclear extracts were centrifuged and the supernatants were carefully removed and transferred to pre-chilled fresh microfuge tube.

For detecting total and phosphorylated IκBα and CaM levels, whole cell extracts were prepared. In brief, cells were lysed using lysis buffer (50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM NaF, 100 mM sodium orthovanadate and 1 mM EGTA) followed by centrifugation for 20 min at 12300 rpm at 4°C. Supernatents were collected and used as whole cell extracts. The freshly prepared cytoplasmic or nuclear or whole cell extracts were either stored at –70°C or they were used for protein estimation by bicinchoninic acid (BCA) method following manufacturer's protocol (Pierce, Rockford, IL).

Equal amounts of the extracts were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoretic transfer, the nitrocellulose membranes were incubated with mouse Ab to CaM (Millipore Bedford, MA) or rabbit Ab to either phosphorylated or total IκBα (Cell Signaling Technology, Beverly, MA) or rabbit Ab to p50 or p65 or c-rel transcription factor (Santa Cruz Biotechnology). Membranes were washed and incubated with either anti-mouse or anti-rabbit immunoglobulin (Ig) coupled to HRP (Sigma-Aldrich). Bound enzyme was detected by enhanced chemiluminescence following the manufacturer's protocol (ECL; GE Healthcare, Little Chalfont, UK) as described earlier (Khan et al., 2006). Equal loading of protein was confirmed by measuring the β-actin level in the same extract by immunoblotting.

2.10. Flow cytometric evaluation of phospho-p38 MAPK

The RAW 264.7 macrophages (1-2 x 10^6) were treated with various concentrations of NAC for 45 min and then fixed with 1.5% formaldehyde (Sigma-Aldrich). After washing, permeabilization was carried out with freshly prepared ice-cold methanol (90% for 30 min) on ice. Cells were washed twice in staining buffer (0.5% BSA in PBS) and incubated with mouse Ab (100 times diluted in staining buffer) to phospho-p38 (pp38) MAPK (Cell Signaling Technology) for 30 min at 37°C. Cells were washed in staining buffer and

probed with anti-mouse-FITC conjugate (Sigma-Aldrich). Flow cytometry was carried out on BD FACSVantage SE (Becton Dickinson, San Jose, CA). The post-flow cytometric data were analyzed using CellQuest data analysis software (Becton Dickison).

2.11. EIA for measuring CaM level

An EIA method involving competitive binding of anti-CaM monoclonal Ab (mAb) (Millipore) was used to measure cytoplasmic CaM levels as described earlier (Khan et al., 2006; Padma and Subramanyam, 2002). Briefly, RAW 264.7 macrophages were either left untreated or treated with various concentrations of NAC for 1 h. In some experiments, RAW 264.7 macrophages were pretreated with 10 µM SB203580 for 30 min followed by treatment with 20 mM NAC. After 1 h, whole cell extracts were prepared and incubated with mouse monoclonal anti-CaM Ab (Millipore) at a ratio of 10:1 and transferred to EIA plates that were previously coated with recombinant CaM (Millipore) protein (10 ng/well). After incubation for 2 h at 37°C, plates were washed with PBS containing 0.05% (v/v) Tween-20 (Sigma-Aldrich) and incubated with goat anti-mouse Ig HRP (Sigma-Aldrich) for 1 h. HRP activity was detected by using *o*-phenylenediamine tetrahydrochloride (Sigma-Aldrich) at 0.5 mg/ml in citrate-phosphate buffer (pH, 5.4) containing 1 µl/ml H_2O_2 (Qualigens). The reaction was terminated using 1 N H_2SO_4 and the absorbance values were measured at 492 nm. CaM levels in the test samples were expressed as the fold change over the untreated control (Khan et al., 2006).

2.12. Measurement of endogenous ROS level

The intracellular ROS level was measured using a fluorescent dye, 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich) as described earlier (Khan et al., 2007). Briefly, cells (3-5 x 10^5) were treated with either varying concentrations of NAC for 10-15 min or a fixed concentration of 3 mM NAC for various time periods. Cells were next incubated with 5 μM DCFH-DA for 15 min in dark. The stained cells were analyzed on a Becton Dickinson flow cytometer (BD FACSVantage SE).

2.13 Immunoprecipitation (IP) assay

The immunoprecipitation assay was carried out as described earlier (Antonsson et al., 2003) with slight modifications. The whole cell extracts prepared from various experimental groups were incubated with 4 μg/ml of anti-CaM Ab (Sigma-Aldrich) or anti SOCS1 Ab for 3 h at 4°C with gentle rocking. Fifteen μl of protein A/G-Sepharose (Santa Cruz Biotechnology) was added to each sample and incubated further for 2 h at 4°C with gentle rocking. Beads were washed extensively with IP buffer containing 25 mM HEPES, 0.1 M NaCl, 10% glycerol, 0.05% Triton X-100, and 2 mM EDTA. After the final wash, beads were re-suspended with 5X SDS sample buffer and heat boiled at 95-100°C for 2-5 min and then samples were loaded on 10% SDS-PAGE gel. Co-

immunoprecipitated c-rel or p65 or p50 or CaM was detected by Western blotting using anti-c-rel or anti-p65 or anti-p50 or anti-CaM antibody as discussed in section 2.9.

2.14. Transient transfection assay

The phosphorylation-defective IκBα (ΔIκBα) plasmid construct and the dominant-negative mutant of p38 (DNp38) construct were kind gifts from Jurgen Heesemann (Max von pettenkoffer-Institute fur Hygiene and Medizinische Microbiologie, Munchen, Germany) and Jiahuai Han (Scripps Research Institute, La Jolla, CA, USA) respectively. All the plasmid constructs were transfected in RAW 264.7 cells using the cationic lipid suspension Lipofectamine 2000 (Invitrogen). Expression vector without any insert was used as negative control. For transient transfection, about 2-3 × 10^6 cells were washed and resuspended in Opti-MEM (Invitrogen). Next, 10 µg DNA and 5 µl Lipofectamine 2000 were separately incubated in 50 µl of Opti-MEM and incubated for 15 min. The two preparations were mixed slowly and kept for 30 min with intermittent mixing. The complex was added drop wise at different places on the culture plate. Complete media with 20% FBS was added after 5-6 h. After 24 h of transfection, cells were either left untreated or treated with 3 or 20 mM NAC. Cells were either harvested after 1 h for c-rel Western blotting or cultured for 48 h to measure IL-12 p40 production. In some experiments, RAW 264.7 macrophages were transfected with 100 nM of control-siRNA or SOCS1-specific siRNA (both from Santa Cruz Biotechnology). After 24 h, cells were

treated with medium or 3 mM NAC and harvested either after 1 h for measuring SOCS1 mRNA levels by semi-quantitative RT-PCR or for detecting the nuclear p65 levels by Western blotting or cultured for 48 h to estimate TNF-α production by EIA and nitric oxide (NO) production by Griess reaction.

2.15. Nitrite estimation

The accumulated nitrite resulting from NO production by the stimulated macrophages in culture was measured using the Griess reaction (Mukhopadhyay et al., 1999; Mukhopadhyay et al., 2004). The assay was performed in 96-well plates using equal volumes of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine (1:1) in 2.5% orthophosphoric acid) and sample. The plates were read at 550 nm absorbance. Nitrite concentrations were calculated based on a standard curve read from a prepared standard solution of sodium nitrite.

2.16. Measurement of intracellular reduced glutathione (GSH) and oxidized glutathione (GSSG) levels

GSH and GSSG levels were measured by enzymatic recycling assay as described earlier (Rahman et al., 2006). Briefly, RAW 264.7 macrophages (3 x 10^6 cells/ml) were treated with varying concentrations of NAC for 1 h and suspended in ice cold extraction buffer

(1% Triton X-100 and 0.6% sulfosalicylic acid) and lysed by repeated freezing and thawing. The extracts were centrifuged 10400 rpm for 5 min at 4°C and the supernatants were used to estimate GSH and GSSG levels. To measure total glutathione content, 50 µl of the each sample as well as GSH (Sigma-Aldrich) or GSSG (Sigma-Aldrich) standards were added in the microtiter plate. Hundred microliters of glutathione assay mix solution containing 1.66 mg/ml NADPH (Sigma-Aldrich), 1.66 mg/ml DTNB (5,5'-dithio-bis (2-nitrobenzoic acid) (Sigma-Aldrich) and 2 units/ml glutathione reductase (GR) (Sigma-Aldrich) were added and then the rate of change in absorbance was measured at 405 nm. To assay GSSG, the GSH present in the sample was derivatized by adding 2 µl of 2-vinylpiridine and 6 µl of triethanolamine to a 100 µl aliquot of supernatant. After 1 h of incubation at 25°C, GSSG was measured in the same way as GSH was quantified. The amount of GSH and GSSG for each sample was determined from the standard curve.

2.17. Lymphocyte proliferation assay

Peripheral blood was obtained from TB patients reported at the DOTS (Directly Observed Treatment – Short-course) Clinic of Mahavir Hospital and Research Centre, Hyderabad, India. The diagnosis of the TB patients was confirmed by tuberculin skin test, radiographic examination, observation of acid-fast bacilli in sputum and clinical symptoms. These patients were negative for HIV virus. The PBMCs from heparinized blood samples from TB patients (*n = 33*) were isolated using gradient centrifugation in

Ficoll-Hypaque (Sigma-Aldrich) solution as described earlier (Chakhaiyar et al., 2004) and prepared at 3 x 10^6 cells/ml in RPMI-1640 (Invitrogen) medium containing 10% FBS (Invitrogen) and antibiotics (Invitrogen). Cell suspensions (3 x 10^5/200 µl/well) were dispensed into 96-well, flat-bottom microtiter plates (Nunc, Roskilde, Denmark) and maintained at 37ºC in 5% CO_2 incubator. PBMCs from various groups were treated with a fixed concentration of NAC (3 mM) and activated in the presence of 10 µg/ml PPD or BCG (1 x 10^5).

In some experiments, macrophage population (MDM) from PBMC was purified as described in the 'Materials and Methods', section 2.3. The adherent cells were greater than 90% macrophages by staining with CD11b Ab. The macrophages were pretreated for 2 h with 3 mM NAC. The cells were washed and used as APCs. Autologous T-cells were purified from non-adherent fraction of the buffy coat that was used to purify MDM, using nylon wool column as described earlier (Raiter et al., 2000). The non-adherent cells were incubated in prewashed nylon wool at 37^{o}C in 5% CO_2. After 60 min, the nylon wool column was washed twice with 10 ml of medium, and the unabsorbed T-cells were collected and counted. Proliferation assays were performed using purified T-cells added at a concentration of 3 x 10^5 cells/200 µl/well with autologous macrophages added at 1 x 10^5 cells/200 µl/well. Cell suspensions (200 µl/well) were dispensed into 96-well, flat-bottom microtiter plates (Nunc) and cultured in the presence of BCG as recall antigen. After 4 days culture supernatants were harvested for estimating IFN-γ and IL-5 cytokines secreted in the culture supernatants by EIA. T cell proliferation was measured

by MTT assay after 4 days. The bioethics committee of Mahavir Hospital and Research Centre and CDFD approved the present study and informed consent was obtained from all the subjects.

2.18. Immunization of mice

BALB/c mice (n = 3) were immunized with BCG subcutaneously at two sides as 0.5 X 10^5/50 µl/inoculum under the cover of NAC. NAC was injected intraperitoneally as 150 mg/kg (Victor et al., 2003) at day -1, 0, +1, +2, +3, +4, and +5. Control groups (n = 3) received PBS alone. After 10 days mice were sacrificed and spleens were harvested. Spleen cells were homogenized and red blood cells were lysed by the addition of 9 ml of sterile distilled water followed by 1 ml of sterile 10X PBS. The cells were then washed twice with Hank's balanced salt solution (HBSS) and the total number was counted by haemocytometer. Splenic cells were resuspended in complete DMEM (Invitrogen) medium containing 10% FBS (Invitrogen) and antibiotics (Invitrogen) and cultured at 37°C as 3 x 10^5 cells/200 µl/well in 96-well plate tissue culture plate with BCG (3 x 10^5). Culture supernatants were harvested after 60 h of culture to measure IL-10 and IFN-γ by EIA using the protocol provided by the manufacturer (BD Biosciences Pharmingen). The splenic T cell proliferation was assayed after 96 h of culture by MTT assay.

2.19. Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons were made using either Student *t* or by one-way ANOVA followed by Tukey's honestly significant test. The value of $p<0.05$ was considered statistically significant.

Chapter 3 - Results

3.1. Intracellular glutathione-redox balance affects IL-12 p40/p70 induction in macrophages

To examine the effect of intracellular glutathione-redox balance on cytokine production in macrophages, the RAW 264.7 macrophages were treated with various concentrations of NAC, a known precursor of glutathione (Deneke and Fanburg, 1989) to alter the GSH and the GSSG concentrations and the GSH/GSSG ratio. The cells were treated with increasing concentrations (0.3-20 mM) of NAC for 1 h and intracellular glutathione-redox status in these macrophages was quantified following enzymatic recycling assay. It was observed that treatment of NAC resulted in increased intracellular GSH in a dose-dependent manner upto a concentration of 3 mM (Fig. 3.1*A*), but the intracellular GSSG levels were not significantly changed within this range of NAC (Fig. 3.1*B*). However, higher concentrations of NAC (10 or 20 mM) increased GSSG levels by about 3 or 4 fold when compared with the medium-treated control macrophages (Fig. 3.1*B*). Intracellular glutathione-redox balance was indicated as GSH/GSSG (Mukherjee et al., 2007). The GSH/GSSG balance showed predominantly a reducing state when macrophages were treated with NAC upto 3 mM due to increased levels of intracellular GSH (Fig. 3.1*C*, compare bar 4 with bar 1). However, the redox balance shifts towards more oxidizing state at higher concentrations of NAC due to increase in intracellular GSSG level (Fig. 3.1*C*, compare bar 6 with bar 1).

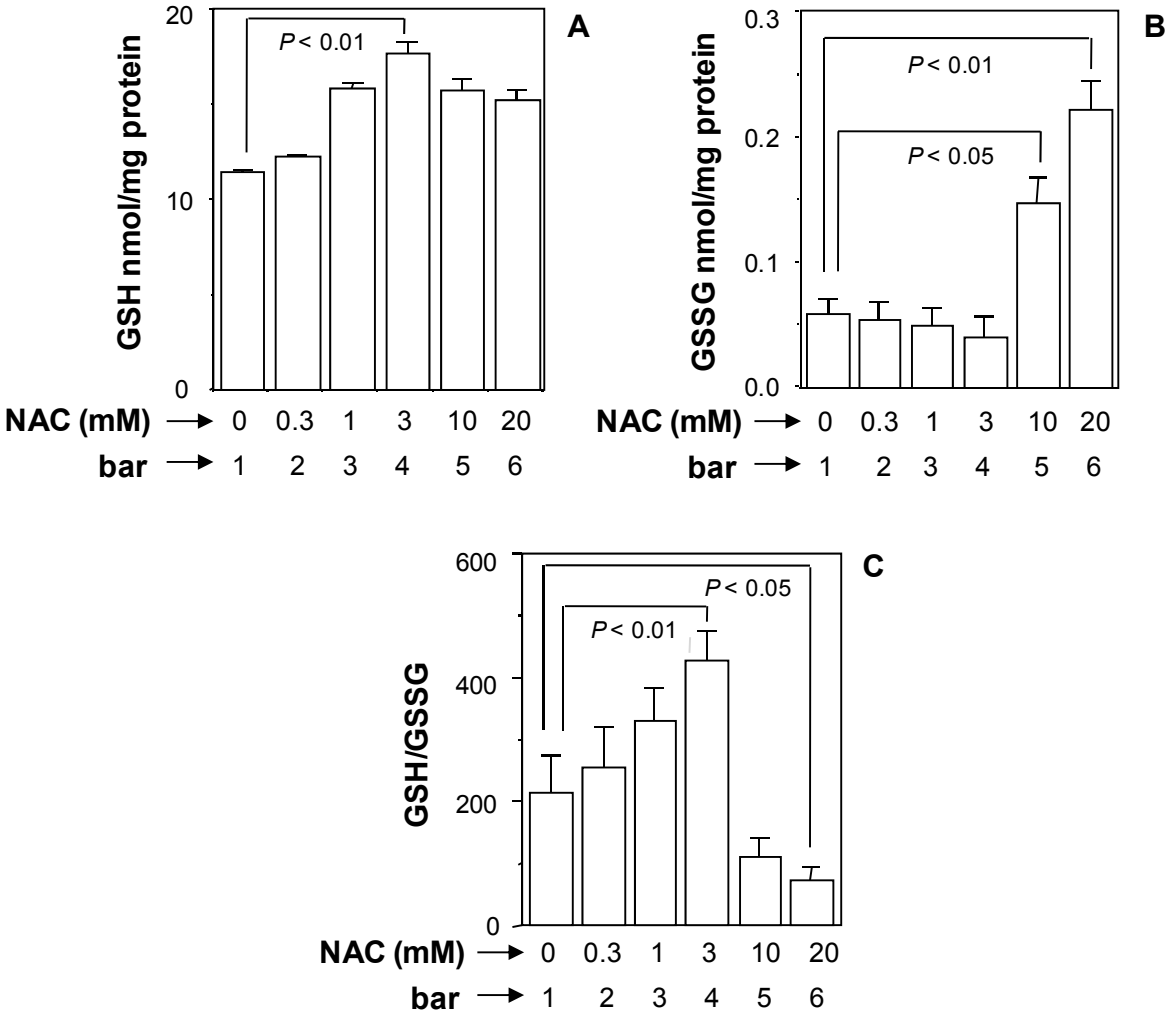

Figure 3.1. NAC modulates the intracellular glutathione-redox status in dose dependent manner. The RAW 264.7 macrophages were treated with various concentrations of NAC for 1 h. Cells were harvested and lysed and the intracellular concentrations (mean ± SD) of GSH (*A*), GSSG (*B*) and the GSH/GSSG (*C*) in all groups were quantified following enzymatic recycling assay. Data are representative of 3 independent experiments. *P* value was calculated by Tukey's HSD tes

Further we investigated whether the changes in the intracellular glutathione-redox balance in macrophages can modulate the levels of various innate cytokines like IL-12 and IL-10 and TNF-α. IL-12 is known to be a heterodimeric protein of 70 kDa, composed of two subunits, IL-12 p35 and IL-12 p40 and regulation of biologically active IL-12 p70 is found to be dependent upon transcriptional regulation of the gene encoding the IL-12 p40 subunit (Khan et al., 2006; Trinchieri, 2003). We, therefore, treated RAW 264.7 macrophages with various concentrations of NAC and at 48 h post-treatment, the levels of IL-12 p40, IL-12 p70 as well as IL-10 and TNF-α were measured in the culture supernatants by EIA. It was observed that NAC at low concentrations activated induction of IL-12 p40 (Fig. 3.2*A*) and IL-12 p70 (Fig. 3.2*B*). Maximum induction of IL-12 p40 (Fig. 3.2*A*) and IL-12 p70 (Fig. 3.2*B*) was observed at 3 mM NAC (Figs. 3.2*A* and 3.2*B*, compare bar 4 with bar 1). However, these levels were decreased with subsequent higher concentrations of NAC, i.e., 10 to 20 mM (Figs. 3.2*A* and 3.2*B*, compares bars 5 and 6 with bar 4). The dose-dependent increase of IL-12 p40 in RAW 264.7 macrophages was also reflected at the mRNA level where maximum expression was observed in an environment rich in reduced glutathione (3 mM NAC) (Fig. 3.2*C*).

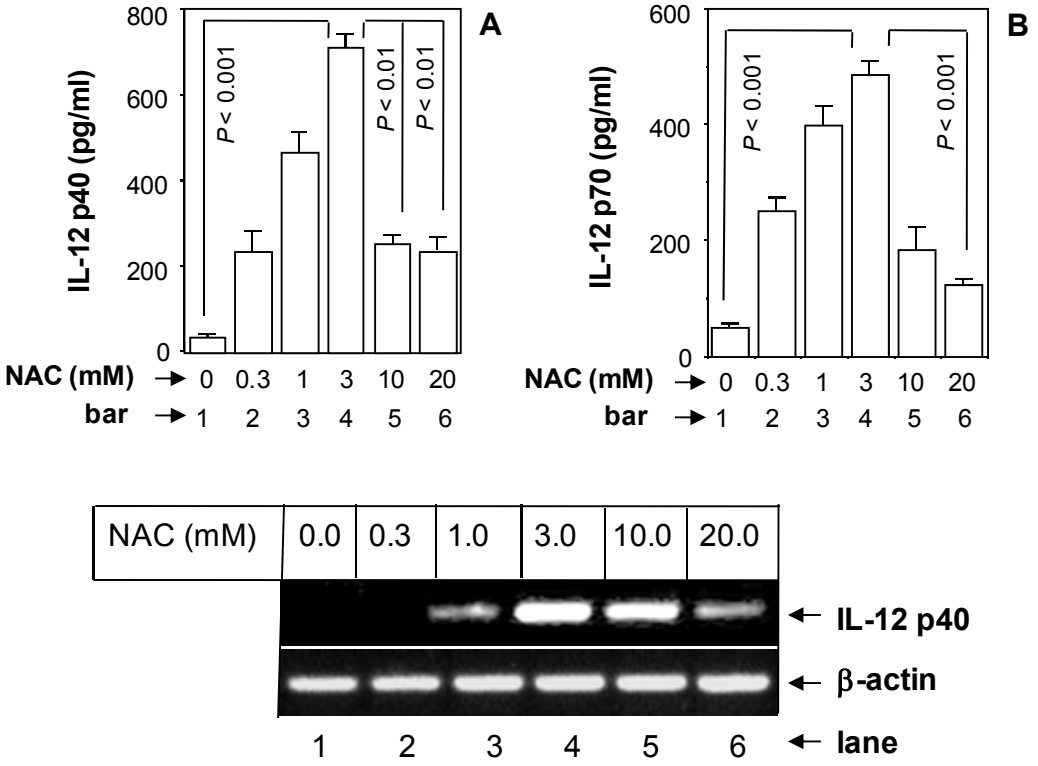

Figure 3.2. IL-12 induction in macrophages is regulated by intracellular glutathione-redox status. The RAW 264.7 macrophages were treated with various concentrations of NAC to change intracellular GSH/GSSG balance. After 48 h, culture supernatants were collected to measure IL-12 p40 (mean ± SD) (*A*) and IL-12 p70 (mean ± SD) (*B*) levels by EIA. In another experiment, RAW 264.7 macrophages were treated with various concentrations of NAC and after 12 h, total RNA was extracted and subjected to semi-quantitative RT-PCR analysis to determine induction of IL-12 p40 mRNA with β-actin used as amplification control. Data are representative of 3 independent experiments. *P* value was calculated by Tukey's HSD test.

The decrease in IL-12 p40 and IL-12 p70 production at higher concentrations of NAC used (10 mM and 20 mM) was not due to cell cytotoxicity, since cell viability remained unchanged at these concentrations as revealed by MTT assay (Fig. 3.3). Next, experiments were carried out to demonstrate that IL-12 p40 induction was directly regulated by the glutathione-redox status. Therefore, RAW 264.7 macrophages were treated with various concentrations of GSH-OEt, a permeable form of glutathione, to alter the intracellular GSH/GSSG ratio. Then, it was checked whether changes in the intracellular GSH/GSSG ratio could influence the IL-12 p40 induction in RAW 264.7 macrophages in a similar ways as observed earlier using NAC (Fig. 3.2). The cells were, therefore, treated with various concentrations of GSH-OEt (1 to 10 mM) and either harvested after 1 h to measure the intracellular GSH and GSSG levels by enzymatic recycling assay as described in the 'Materials and Methods (2.16)' or cultured for 48 h to measure the IL-12 p40 levels secreted in the culture supernatants by EIA. It was observed that exogenous treatment of RAW 264.7 macrophages with GSH-OEt altered the intracellular GSH/GSSG ratio in macrophages in dose-dependent manners (Fig. 3.4*A*) as expected (Deneke and Fanburg, 1989), and similarly influenced IL-12 p40 induction (Fig. 3.4*B*). These data indicate that IL-12 p40 induction in macrophages is directly regulated by the glutathione-redox status.

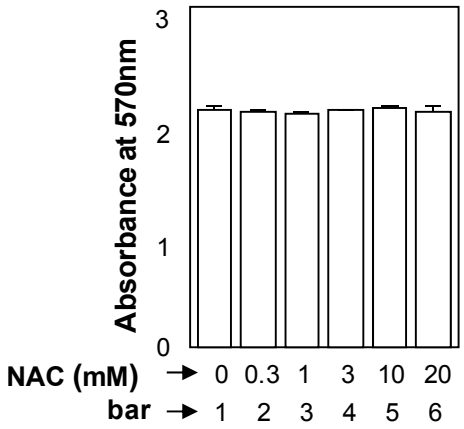

Figure 3.3. NAC does not affect the cell cytotoxicity. The RAW 264.7 macrophages were treated with various concentrations of NAC. After 48 h, the cell cytotoxicity was measured by MTT assay. Data are representative of 3 independent experiments.

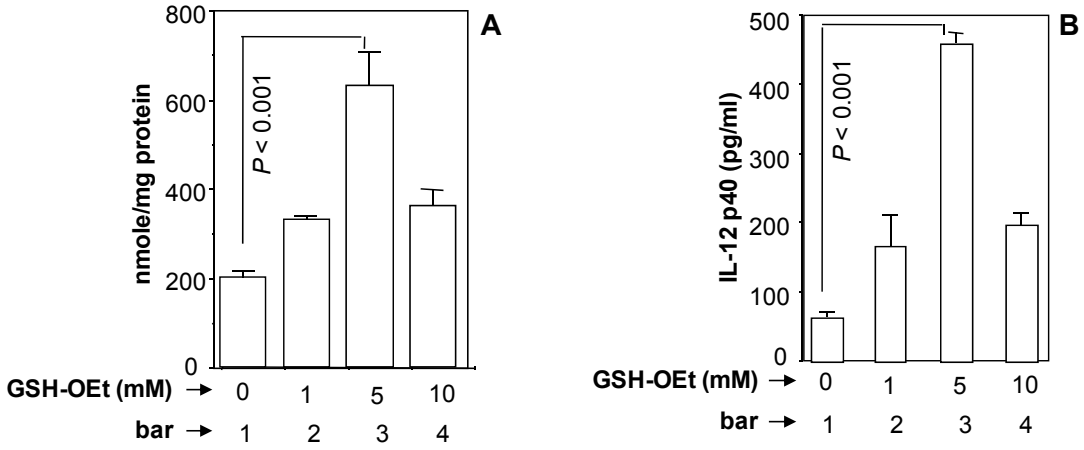

Figure 3.4. Intracellular glutathione-redox of macrophages is changed by the permeable GSH-OEt leading to changes in the IL-12 p40 induction. The RAW 264.7 macrophages were treated with various concentrations of the permeable GSH-OEt. Cells were either harvested after 1 h to measure the intracellular GSH/GSSG (*A*) ratios or cultured for 48 h to quantify IL-12 p40 levels secreted in the culture supernatants by EIA (mean ± SD) (*B*). Data are representative of 3 independent experiments. *P* value was calculated by Tukey's HSD test.

Though NAC is widely used as a modulator of intracellular redox balance some studies have reported that NAC can also have several other pleiotropic effects (DiMari et al., 1997; Wilhelm et al., 1997). Therefore to ascertained the observed effect of NAC is due to the change in the glutathione-redox balance and not due to direct effect of NAC, the RAW 264.7 macrophages were treated with BSO (200 μM), a pharmacological inhibitor of γ-GCS, which inhibits conversion of NAC into GSH or in other words inhibits anti-oxidant properties of NAC. It was expected that in the presence of BSO, NAC will not increase GSH levels. The results indicated that BSO was able to inhibit the intracellular GSH level in macrophages treated with NAC (3 mM), as expected (Deneke and Fanburg, 1989) (Fig. 3.5*A*). BSO treatment results in suppression of IL-12 p40 induction (Fig. 3.5*B*) even when macrophages were treated with 3 mM NAC. These observations indicate that the observed effect is not due to pleiotropic effect of NAC but due to NAC-mediated change in glutathione-redox balance.

A dose-dependent effect of NAC on IL-12 p40 induction was also observed in monocyte-derived macrophages (Fig. 3.6). These results indicate that the effect of NAC on IL-12 p40 transcription was not limited to transformed cell lines only**.**

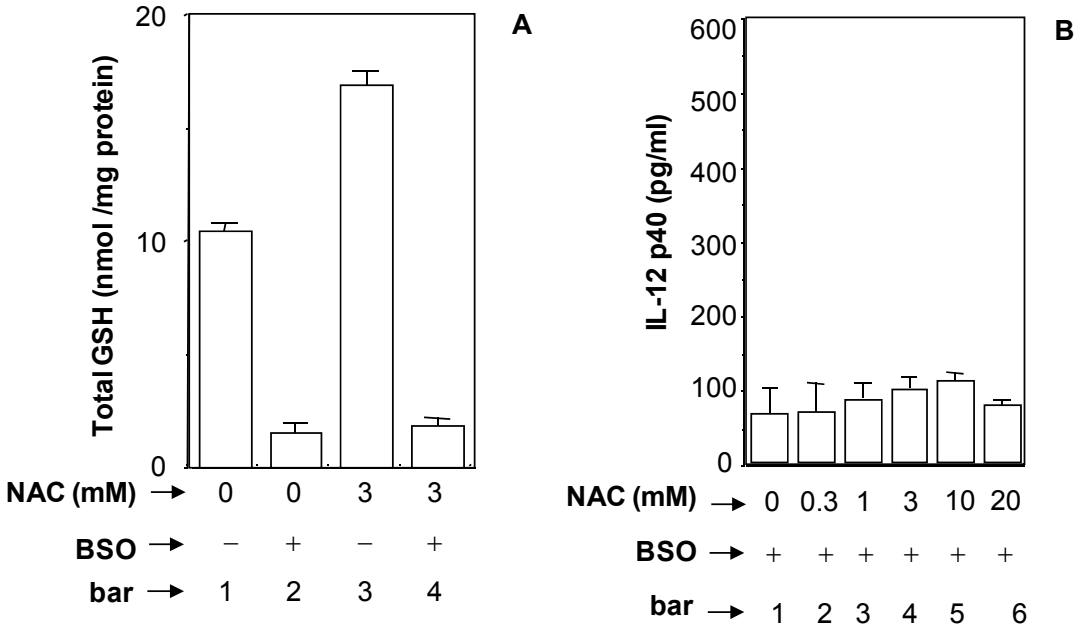

Figure 3.5. BSO, a pharmacological inhibitor of GSH synthesis decreases intracellular GSH level and IL-12 induction in macrophages treated with 3 mM NAC. (*A*), RAW 264.7 macrophages were either left untreated or treated with 3 mM NAC in the absence or presence of 200 µM of BSO. After 1 h, cells were washed, lysed and the intracellular GSH levels (mean ± SD) were quantified following enzymatic recycling assay. (*B*), IL-12 p40 (mean ± SD) was measured in RAW 264.7 macrophages treated with various concentrations of NAC in the presence of BSO. Data are representative of 3 independent experiments.

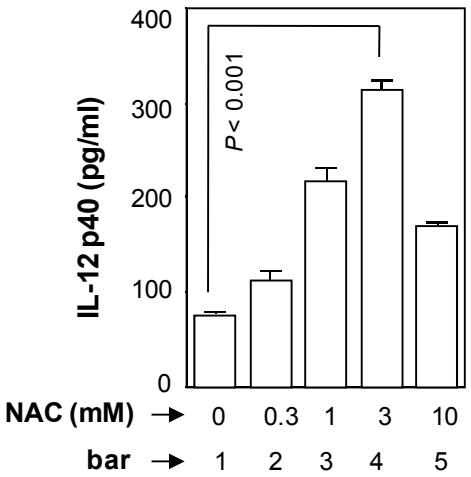

Figure 3.6. NAC affects IL-12 p40 induction in concentration-dependent manner also in monocyte-derived macrophages. Monocyte-derived macrophages were purified from PBMCs from healthy volunteers as described in 'Materials and Methods Section 2.3'. The cells were treated for 48 h with various concentrations of NAC. Culture supernatants were harvested to measure IL-12 p40 levels by EIA. Data are representative of 3 independent experiments. *P* value was calculated by Tukey's HSD test.

These data clearly document that the intracellular glutathione-redox can regulate IL-12 induction in macrophages. To observe the glutathione-redox effect on other innate-cytokines like TNF-α and IL-10, the same culture supernatants that were used to measure IL-12 p40 and IL-12 p70 levels as shown in Figs. 3.2*A* and 3.2*B*, were checked for IL-10 and TNF-α production by EIA. It was observed that there was no change in the TNF-α production although a slight increase in IL-10 was noticed only in the group treated with 20 mM NAC (Fig. 3.7).

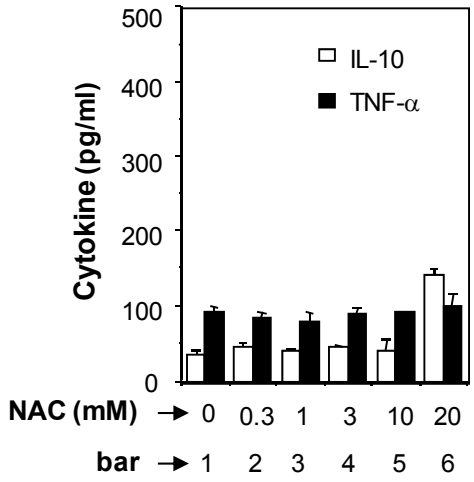

Figure 3.7. Induction of IL-10 and TNF-α in NAC-treated RAW 264.7 macrophages. RAW 264.7 macrophages were treated with various concentrations of NAC. After 48 h culture supernatants were harvested and the amount of IL-10 and TNF-α secreted in the culture supernatants were measured by EIA. Data shown are mean ± SD of 3 different experiments.

3.2. Nuclear c-rel level is regulated by the macrophages glutathione-redox state

Since the intracellular GSH/GSSG balance was found to influence IL-12 p40 induction, the possible molecular mechanisms involved in such regulation were investigated. There are reports which indicate that the redox balance can affect the rel family of transcription factors to a large extent (Toledano and Leonard, 1991). Among the rel factors, the c-rel is known to play a central role in IL-12 p40 transcription (Khan et al., 2006; Sanjabi et al., 2000). Therefore, nuclear c-rel level was first measured in cells treated with varying concentrations of NAC. The RAW 264.7 macrophages were treated with varying concentrations of NAC (ranging from 0.3 mM to 20 mM) for 1 h. The cells were harvested to prepare cytoplasmic and nuclear extracts from each group. The cytoplasmic and nuclear c-rel levels were measured by Western blotting using anti-c-rel Ab. The nuclear c-rel was found to be markedly increased in the cells treated with 3 mM NAC (Fig. 3.8*C*, compare lane 4 with lane 1). However, nuclear c-rel levels were decreased with higher NAC concentrations (10 and 20 mM) (Fig. 3.8*C*, compare lanes 5 and 6 with lane 4). This was correlated well with the cytoplasmic c-rel level (Fig. 3.8*B*). The total c-rel level was not affected by the changes in the GSH/GSSG balance (Fig. 3.8*A*). These data indicate that probably intracellular glutathione balance affects nuclear translocation of the c-rel transcription factor.

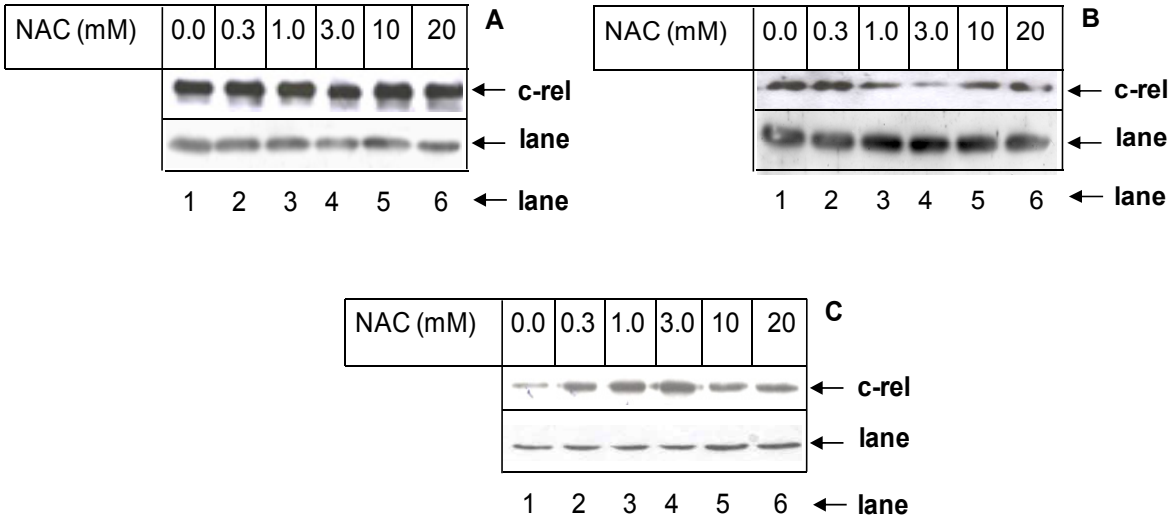

Figure 3.8. Nuclear c-rel level is regulated by the glutathione-redox balance of macrophages. RAW 264.7 macrophages were treated with varying concentrations of NAC as indicated. Cells were harvested after 1 h and c-rel levels were measured in the total cell extracts (*A*), cytoplasmic extracts (*B*) and nuclear extracts (*C*) by Western blotting using anti-c-rel Ab. Data are representative of 3 independent experiments.

The c-rel is known to be controlled upstream by the cytoplasmic IκBα which sequesters c-rel in the cytoplasm (Ghosh et al., 1998; Rahim et al., 2005). During macrophage activation, IκBα is phosphorylated, releasing the cytoplasmic c-rel to translocate to the nucleus. Therefore, it was investigated whether intracellular glutathione-redox has a direct effect on the levels of phosphorylation and degradation of IκBα. Since, NAC at 3 mM concentration increased IL-12 p40 expression (Fig. 3.2*A*), it was speculated that NAC at this concentration will also increase the phosphorylation of IκBα and its subsequent degradation. In a pilot experiment, it was observed that IκBα phosphorylation was highest at 45-60 min post-NAC treatment (Fig. 3.9*A*). Therefore, in the subsequent experiments, levels of IκBα phosphorylation were examined at 45 min after treating cells with various concentrations of NAC by immunoblotting. It has been observed that IκBα phosphorylation was increased by both 1 and 3 mM NAC (Fig. 3.9*B*, compare lanes 3 and 4 with lane 1) suggesting that intracellular GSH/GSSG balance at NAC concentration of 1 or 3 mM probably targets the IκBα phosphorylation and degradation to increase nuclear c-rel level (Fig. 3.8*C*) and IL-12 p40 induction (Fig. 3.2*A*). To confirm this hypothesis, RAW 264.7 macrophages were transfected with phosphorylation-defective IκBα (ΔIκBα) plasmid construct or with backbone vector (PRC/CMV) and IL-12 p40 induction was measured in these macrophages after treatment with 3 mM NAC. It was observed that NAC at 3 mM concentration failed to increase IL-12 p40 in ΔIκBα-transfected cells (Fig. 3.10, compare bar 4 with bar 2) indicating that IκBα plays a role in up-regulation of IL-12 p40 by 3 mM NAC.

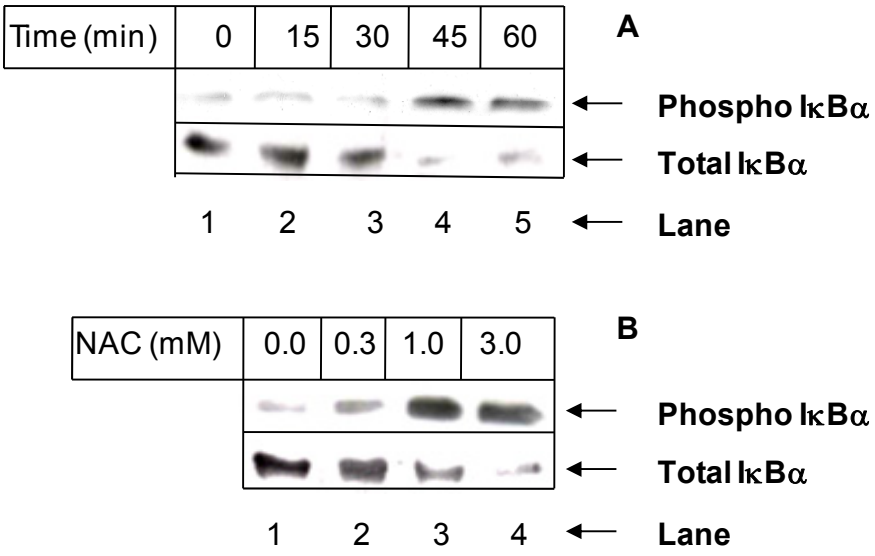

Figure 3.9. Intracellular glutathione-redox has a direct effect on the levels of phosphorylation and degradation of IκBα. RAW 264.7 macrophages were either treated with a fixed concentration of NAC (3 mM) for various time points (*A*) or with different concentrations of NAC for a fixed time of 45 min (*B*). The cells were lysed and total or phosphorylated IκBα was measured by immunoblotting using total or phosphorylated anti-IκBα Ab. Data are representative of 3 independent experiments.

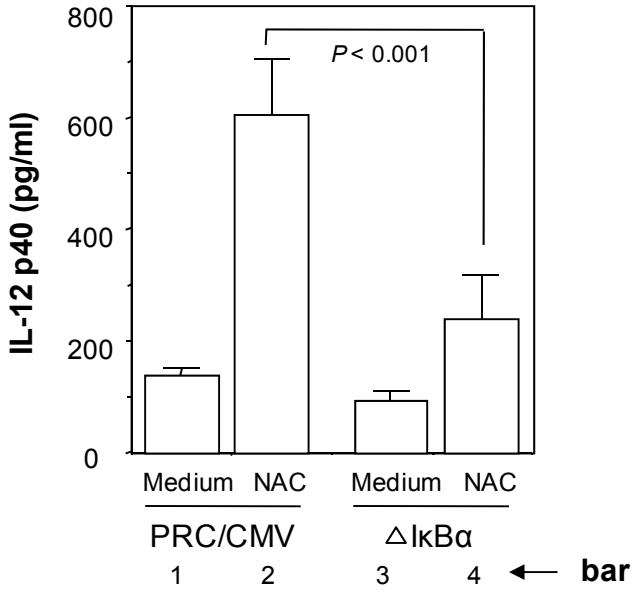

Figure 3.10. The intracellular GSH/GSSG levels at 3 mM NAC concentration targets the IκBα signaling to activate IL-12 p40 induction. RAW 264.7 macrophages were transiently transfected with either phosphorylation-defective IκBα (△IκBα) plasmid construct or with backbone vector (PRC/CMV). Twenty four hours after transfection, cells were treated with medium alone or 3 mM NAC. After 48 h, culture supernatants were harvested to measure IL-12 p40 levels by EIA. Data shown are mean ± SD of 3 different experiments. *P* value was calculated by Student's *t* test.

It is interesting to note that although nuclear c-rel and IL-12 p40 levels were up-regulated in macrophage environment rich in reduced glutathione (treated with 3 mM NAC), their levels were decreased in macrophage environment rich in oxidized glutathione i.e., when treated with 10 and 20 mM NAC. Therefore, it was speculated that the resultant decrease in the level of IL-12 p40 was due to inhibition of IκBα phosphorylation by NAC at these concentrations. However, levels of IκBα phosphorylation were actually increased in these cells (Fig. 3.11, compare lanes 3 and 4 with lane 1). These results hint that the oxidized state of glutathione probably regulates nuclear c-rel levels by targeting different signaling cascades in macrophages.

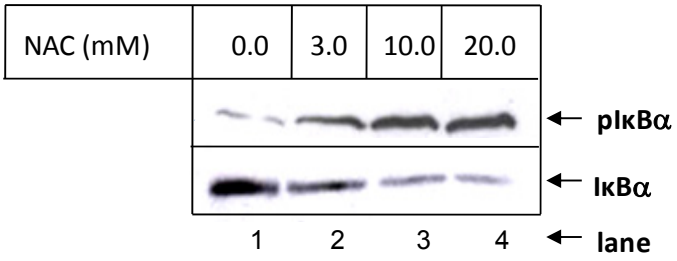

Figure 3.11. Immunoblot analysis of total and phosphorylated IκBα in RAW 264.7 macrophages treated with 10 and 20 mM NAC. RAW 264.7 macrophages were treated with 3, 10 and 20 mM concentrations of NAC for 45 min. The cells were lysed and total or phosphorylated IκBα was measured by immunoblotting using total or phosphorylated anti-IκBα Ab. Data are representative of 3 independent experiments.

3.3. Calmodulin (CaM) protein is involved in the regulation of nuclear c-rel and IL-12 p40 by the oxidized glutathione

CaM protein, a highly conserved, ubiquitously expressed, intracellular sensor for calcium (Chin and Means, 2000), is known to interact with IκBα-released c-rel and sequester c-rel in the cytoplasm inhibiting its nuclear transport (Antonsson et al., 2003; Khan et al., 2006). Since nuclear c-rel level was poorly expressed (Fig. 3.8*C*), although increased IκBα phosphorylation was observed in cells treated with 10 and 20 mM NAC (Fig. 3.11), it was speculated that macrophages rich in GSSG (treated with 10 and 20 mM NAC) probably expressed higher levels of CaM. To validate this hypothesis, RAW 264.7 macrophages were treated with various concentrations of NAC for 1 h and levels of CaM were measured by EIA (Khan et al., 2006) as well as by immunoblotting using anti-CaM Ab. It was observed that CaM expression was increased in macrophages treated with 10 mM and 20 mM NAC (Fig. 3.12*A*, bars 4 and 5 and Fig. 3.12*B*, lanes 5 and 6). CaM expression was not affected by 3 mM NAC (Fig. 3.12*A*, bar 3 and Fig. 3.12*B*, lane 4) indicating that the oxidized glutathione plays important role in increasing CaM level in macrophages.

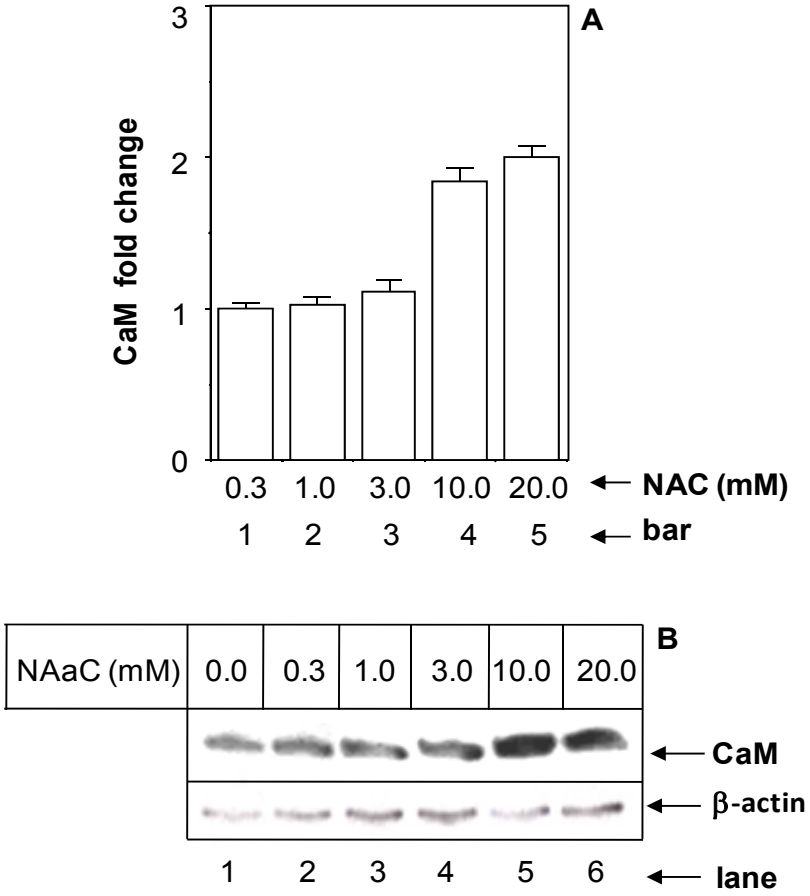

Figure 3.12. Calmodulin level is upregulated in macrophages rich in oxidized glutathione. The RAW 264.7 macrophages were treated with various concentrations of NAC. After 1 h, cells were harvested and whole cell extracts were prepared. (*A*), CaM levels (mean ± SD) in the cell extracts were determined by EIA as described in "Materials and Methods" and expressed as the fold changes over unstimulated control. (*B*), CaM levels in these groups were also measured by Western blotting using anti-CaM Ab. Data are representative of 3 independent experiments.

Next, it was investigated whether CaM was directly involved in the suppression of nuclear c-rel and IL-12 p40 by the intracellular GSSG. RAW 264.7 macrophages were therefore, treated with 20 mM NAC in the absence or presence of 5 μM TFP, a known pharmacological inhibitor of CaM activity (Kamath et al., 1993). Both c-rel (Fig. 3.13*A*, compare lane 4 with lane 2) and IL-12 p40 (Fig. 3.13*B*) levels were increased in cells treated with 20 mM NAC along with TFP as compared to the macrophages treated with 20 mM NAC alone. These data clearly indicate that oxidized glutathione targets the CaM signaling to inhibit c-rel translocation to nucleus, and consequently IL-12 p40 gene transcription levels are reduced. To further confirm involvement of CaM in such regulation, levels of c-rel bound to CaM was directly measured by immunoprecipitation. The whole cell extracts were prepared from RAW 264.7 macrophages treated with medium, 3 mM and 20 mM NAC and were immunoprecipitated using anti-CaM Ab and then immunoblotted with anti-c-rel Ab. Increased amount of c-rel was co-immunoprecipitated in the group treated with 20 mM NAC as compared to groups treated with either medium alone or 3 mM NAC (Fig. 3.13*C*). These observations indicate a direct role of CaM in the GSSG-mediated regulation of IL-12 p40.

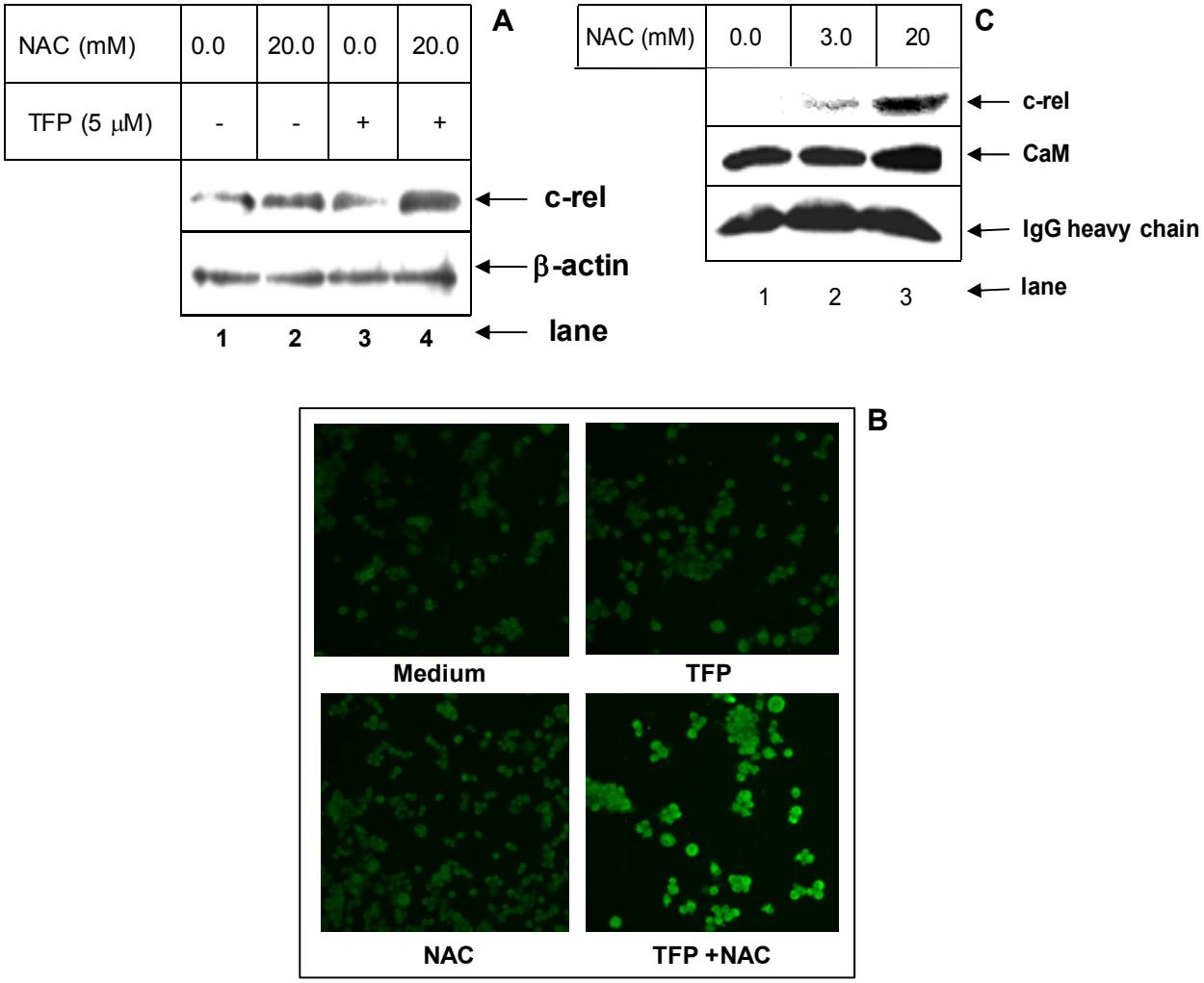

Figure 3.13. The calmodulin (CaM) protein plays an important role in the GSSG-mediated regulation of c-rel and IL-12 p40. The RAW 264.7 macrophages were treated with 20 mM NAC in the absence or presence of 5 μM TFP (a known pharmacological inhibitor of CaM activity). The nuclear c-rel was measured by Western blotting using anti-c-rel Ab (*A*) and the intracellular IL-12 p40 levels was measured by immunofluorescence microscopy (*B*). Again, the whole cell extracts prepared from 3 mM and 20 mM NAC were incubated with anti-CaM Ab for 3 h at 4°C and then protein A/G-Sepharose was added to the mixture and was further incubated for 2 h at 4°C. Co-immunoprecipitated c-rel was detected by Western blotting using anti-c-rel Ab (*C, top lane*). The same preparation was also used to detect CaM level using anti-CaM Ab respectively (*C, middle lane*). Data are representative of at least 3 experiments.

Recently, our lab has shown that the reactive oxygen species (ROS) play important roles in the redox-mediated regulation of CaM expression (Khan et al., 2006). Therefore, it was speculated that NAC at 20 mM concentration probably increased ROS levels which in turn downregulated nuclear c-rel and IL-12 p40 via CaM. To test this, RAW 264.7 macrophages were treated with 20 mM NAC and ROS production was measured at various time points by flow cytometry using the fluorescent dye, 2', 7'-dichlorofluorescein diacetate (DCFH-DA). It was found that NAC at 20 mM concentration decreased intracellular ROS levels in time-dependent manner (Fig. 3.14*A*), which could be due to the direct quenching effect of NAC. Results shown in figure 3.14*B* indicate that NAC inhibits intracellular ROS in dose-dependent manner. These data indicate a ROS-independent mechanism of IL-12 p40 regulation by the oxidized glutathione *via* CaM-c-rel signaling.

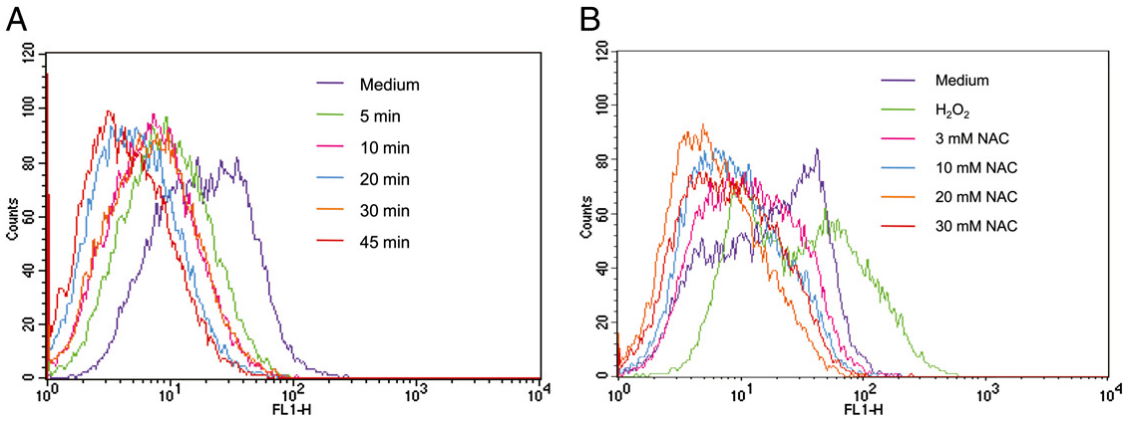

Figure 3.14. NAC inhibits intracellular ROS in time and concentration-dependent manner. The RAW 264.7 macrophages were treated with either 3 mM NAC for various time points (*A*) or with various concentrations of NAC for a fixed time point of 10-15 min (*B*). Cells were then incubated with 5 µM DCFH for 15 min in dark and the stained cells were analyzed on a Becton Dickinson flow cytometer. Data are representative of 4 independent experiments.

3.4. The p38 MAPK is involved in the oxidized glutathione-mediated regulation of c-rel and IL-12 p40 in RAW 264.7 macrophages

In the previous section, it has been observed that NAC at higher concentrations (10 mM and 20 mM) increases CaM levels (Fig. 3.12 and 3.13) independent of ROS (Fig. 3.14) suggesting possible involvement of some other stress-related signaling pathways in such regulation. Recently, it has been reported that one of the signaling pathways regulating IL-12 p40 upstream of CaM could be the p38 MAPK (Boddupalli et al., 2007). Therefore, it was assumed that up-regulation of CaM expression by oxidized state of glutathione could be due to activation of p38 MAPK pathway. To testify this assumption, first the time point at which 20 mM NAC induced highest levels of phosphorylation of p38 MAPK was determined by Western blot analysis using anti-phospho p38 MAPK Ab and a treatment of 45 min was found to be optimum (Fig. 3.15*A*). When the levels of phosphorylated p38 MAPK were compared in macrophages treated with various concentrations of NAC for 45 min, it was observed that p38 MAPK phosphorylation was significantly increased only when NAC was used at 10 mM and 20 mM concentrations (Fig. 3.15*B*). These data indicate that p38 MAPK phosphorylation increases in macrophages when the intracellular GSSG levels are higher.

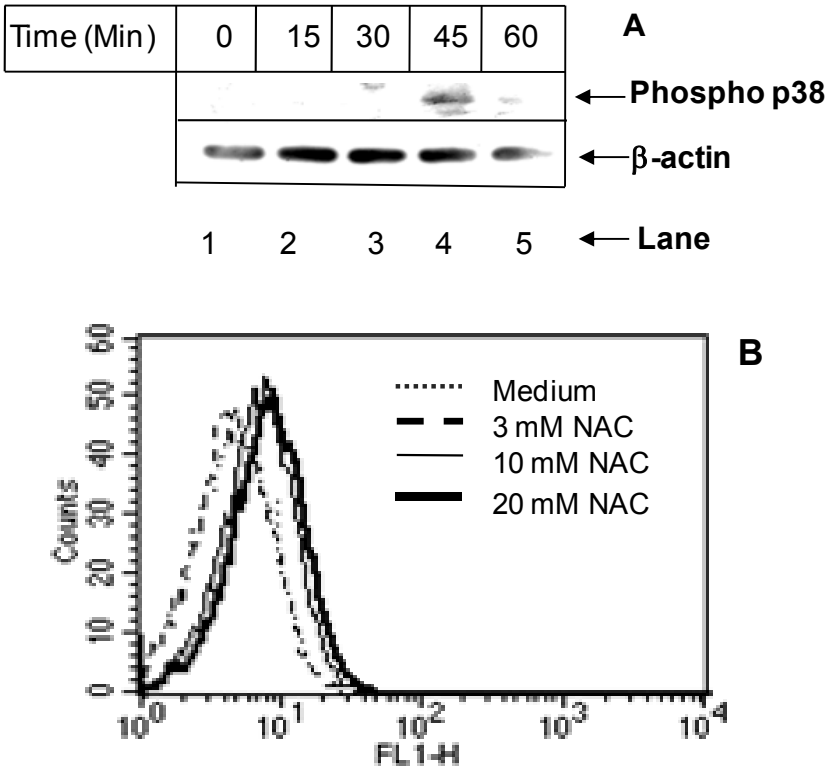

Figure 3.15. The p38 MAPK is activated in RAW 264.7 macrophages rich in oxidized glutathione. (*A),* The RAW 264.7 macrophages were treated with a fixed concentration of NAC (20 mM) for various time points and lysed to detect phospho p38 MAPK levels by Western blotting using anti-phospho p38 MAPK Ab. (*B*), In another experiment, RAW 264.7 macrophages were treated with different concentrations of NAC for a fixed time of 45 min. The cells were then fixed with 1.5% formaldehyde and permeabilized and incubated with mouse anti-phospho p38 MAPK Ab followed by incubation with anti-mouse FITC. The fluorescence was measured by flow cytometry. Data are representative of 3 independent experiments.

To corroborate direct involvement of p38 MAPK in the regulation of CaM as well as IL-12 p40 in macrophages rich in GSSG, experiments were carried out where the p38 MAPK was inhibited using the pharmacological inhibitor of p38 MAPK, SB203580 (Boddupalli et al., 2007) and checked whether CaM expression was downregulated leading to increased nuclear translocation of c-rel and induction of IL-12 p40. Therefore, RAW 264.7 macrophages were either left untreated or pre-treated with 10 μM SB203580 for 30 min and then treated with 20 mM NAC. Nuclear c-rel levels were measured after 1 h by immunoblotting and intracellular IL-12 p40 expression was checked after 6 h by immunofluorescence microscopy. Levels of CaM in whole cell extracts were measured after 1 h by EIA. It was found that presence of SB203580 increased nuclear c-rel (Fig. 3.16*A*, compare lane 4 with lane 2) as well as intracellular IL-12 p40 (Fig. 3.16*B*) expression in macrophages even treated with 20 mM NAC. SB203580 decreased CaM level in macrophages treated with 20 mM NAC (Fig. 3.17). These results indicate that p38 MAPK plays an important role in the GSSG-mediated regulation of CaM expression in macrophages which subsequently controls IL-12 p40 induction.

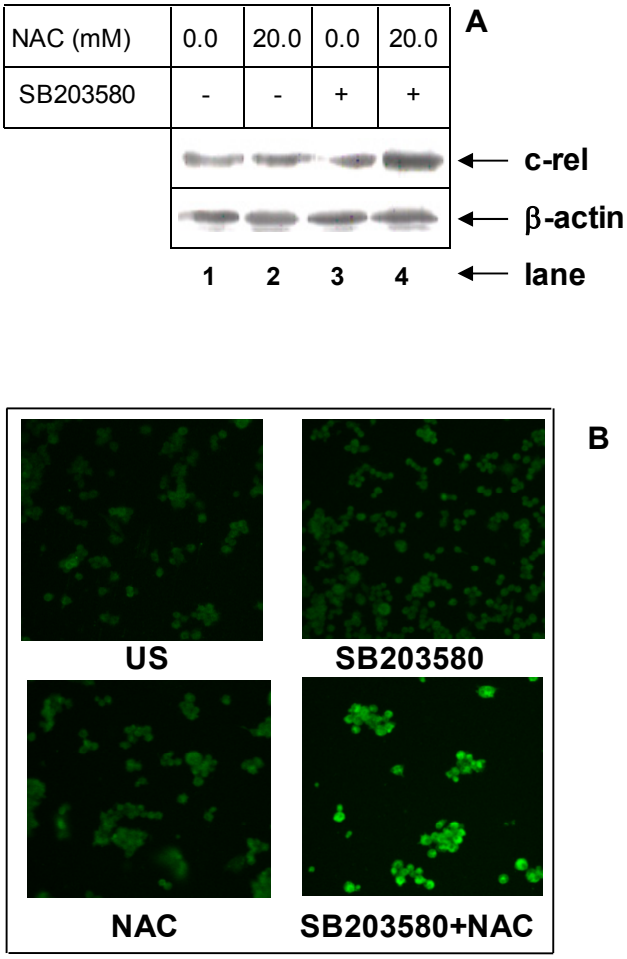

Figure 3.16. The p38 MAPK is involved upstream of CaM in the regulation of c-rel and IL-12 p40 in RAW 264.7 macrophages rich in oxidized glutathione. The RAW 264.7 macrophages were treated with 20 mM NAC in the absence or presence of 10 µM SB203580. Cells were either harvested to detect nuclear c-rel levels by Western blotting using anti-c-rel Ab (*A*) or for measuring the intracellular IL-12 p40 levels by immunofluorescence microscopy (*B*). Data are representative of 3 independent experiments.

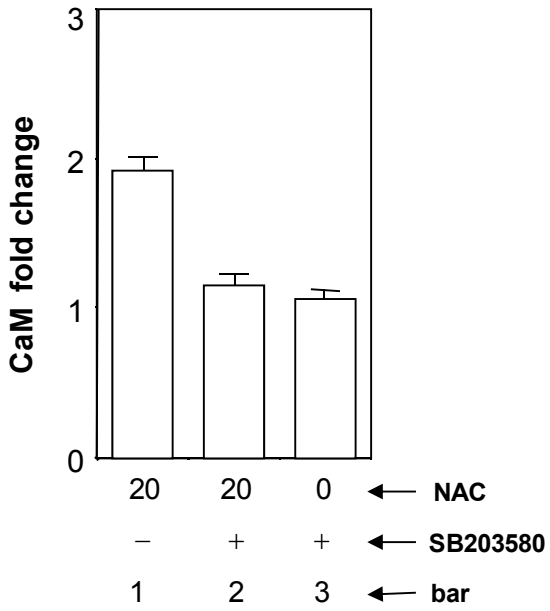

Figure 3.17. The pharmacological inhibitor of p38 MAPK, SB203580 inhibits CaM level in RAW 264.7 macrophages treated with 20 mM NAC. The RAW 264.7 macrophages were treated with 20 mM NAC in the absence or presence of 10 μM SB203580. After 1 h, cells were lysed and the CaM level (mean ± SD) in each group was determined by EIA as described in the "Materials and Methods" and expressed as the fold changes over unstimulated control. Data are representative of 3 independent experiments.

To further confirm the regulatory role of p38 MAPK on c-rel-IL-12 p40 signaling in macrophages rich in GSSG, dominant-negative mutant of p38 (DNp38) was used. The RAW 264.7 macrophages were, therefore, transfected with either backbone vector (pCDNA3) or with DNp38 plasmid and at 24 h post-transfection, both the groups were treated with 20 mM NAC. The cells were either harvested after 1 h to measure nuclear c-rel levels or cultured for 48 h to measure the IL-12 p40 levels secreted in the culture supernatants. It was observed that there was an up-regulation of nuclear c-rel (Fig. 3.18*A*, compare lane 4 with lane 2) as well as IL-12 p40 (Fig. 3.18*B*, compare bar 4 with bar 2) in the DNp38-transfected group as compared to the group transfected with the pCDNA3 backbone vector alone.

All these results support the view that increase in GSSG levels in macrophages resulted in activation of p38 MAPK, in turn up-regulating CaM expression which subsequently downregulated IL-12 p40 induction by directly binding and sequestering c-rel in the cytoplasm and inhibiting its translocation to the nucleus.

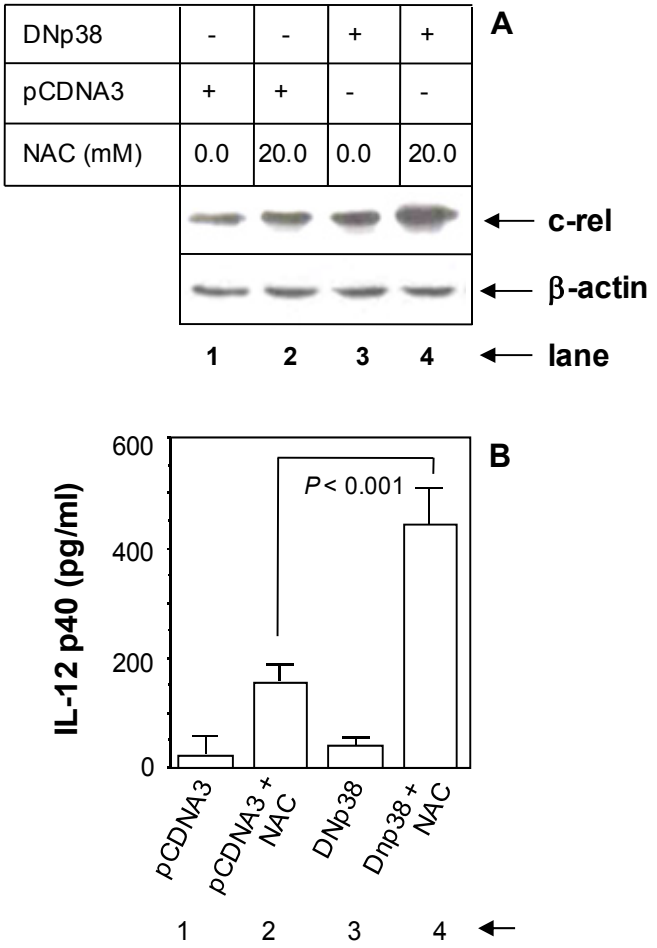

Figure 3.18. Presence of dominant negative mutant of p38 MAPK increases both c-rel and IL-12 p40 in RAW 264.7 macrophages treated with 20 mM NAC . The RAW 264.7 macrophages were transiently transfected with either the DNp38 or the backbone vector pCDNA3. Cells were treated with 20 mM NAC and either harvested after 1 h to examine the nuclear c-rel by Western blotting (*A*) or cultured for 48 h to measure IL-12 p40 (mean ± SD) by EIA (*B*). Data are representative of 3 independent experiments. *P* value was calculated by Student's *t* test.

3.5. NAC at 3 mM concentration increases suppressor of cytokine signaling 1 (SOCS1) expression that prevents nuclear translocation of p65 NF-κB in macrophages causing inhibition of TNF-α and NO production

It is known that the c-rel as well as other NF-κB factors like p50 and p65 are all bound to the IκBα in the cytoplasm and are released to translocate to nucleus after IκBα gets phosphorylated and degraded (Hoffmann et al., 2006; Karin and Ben-Neriah, 2000; May and Ghosh, 1997; Rahim et al., 2005). Since increased phosphorylation and degradation of IκBα was noticed at 3 mM NAC concentration (a situation with higher intracellular GSH/GSSG ratio), it was expected that similar to c-rel (Fig. 3.8*C*), more p50 and p65 NF-κB would be available in the nucleus to start transcription of the NF-κB-dependent genes like TNF-α and iNOS (Khan et al., 2007; Mukhopadhyay et al., 1999; Mukhopadhyay et al., 2004; Ziegler-Heitbrock et al., 1993). However, it was found that NAC at 3 mM concentration did not activate TNF-α (Fig. 3.7). Therefore, mechanism responsible for specific suppression of TNF-α and NO but not IL-12 in macrophages treated with 3 mM NAC was examined. It is known that the SOCS1 negatively regulates NF-κB activation by specifically targeting p65 NF-κB through proteasome-mediated degradation (Ryo et al., 2003). SOCS1 was found to directly interact with p65 and enhanced its ubiquitination presumably by its ubiquitin ligase-like activities. However, the p50 levels remain largely unaffected (Ryo et al., 2003). Since, NAC treatment augmented IL-12 synthesis (Fig. 3.2), but not TNF-α (Fig. 3.7) (largely regulated by p65 NF-κB), it was speculated that NAC probably induced SOCS1, which in turn negatively

affected nuclear translocation of p65 NF-κB by virtue of its preferential targeting of p65 to the proteasome (Ryo et al., 2003). Therefore, next the levels of SOCS1 were examined upon treatment with 3 mM NAC or 5 mM GSH-OEt by immunofluorescence microscopy using anti-SOCS1 Ab. It was observed that treatments of RAW 264.7 macrophages with 3 mM NAC (Fig. 3.19*A*) or 5 mM GSH-OEt (Fig. 3.19*B*) increased SOCS1 expression levels.

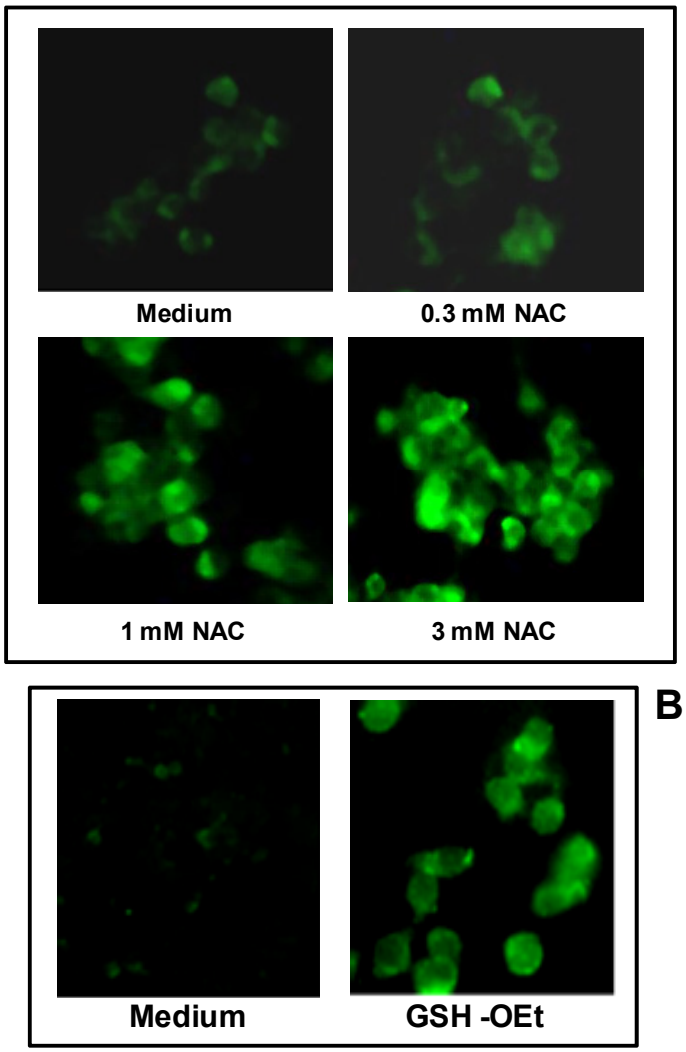

Figure 3.19. SOCS1 protein expression is increased in macrophages treated with 3 mM NAC or 5 mM GSH-OEt. RAW 264.7 macrophages were treated for 1 h with 0.3, 1 or 3 mM NAC (*A*) or 5 mM GSH-OEt (*B*). Cells were washed, fixed and permeabilized and incubated with goat anti-SOCS1 Ab followed by incubation with anti-goat FITC. Intracellular SOCS1 expression was measured by immunofluorescence microscopy. Data are representative of 3 independent experiments.

The increase in SOCS1 level by 3 mM NAC was also checked at mRNA levels by carrying out semi-quantitative reveres-transcriptase PCR. A time-dependent expression profile indicated that 3 mM NAC increased SOCS1 mRNA as early as 15 min time point (Fig 3.20).

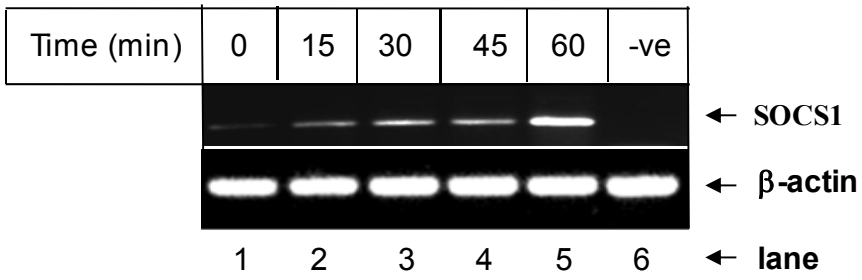

Figure 3.20. Effect of NAC on SOCS1 mRNA expression. Semi-quantitative RT-PCR analysis of SOCS1 mRNA levels in RAW 264.7 macrophages treated with 3 mM NAC at different time points indicated. The mRNA levels were normalized to β-actin mRNA levels. Data are representative of 4 independent experiments

Furthermore, co-immunoprecipitation assay was carried out using anti-SOCS1 Ab to check whether SOCS1 specifically interacts with p65 NF-κB but not with other transcription factors like c-rel and p50 NF-κB. The co-immunoprecipitation assay clearly revealed that SOCS1 strongly interacted with the p65 subunit of NF-κB (Fig. 3.21*A*) and as a result nuclear p65 levels were barely detectable (Fig. 3.21*B*). However, SOCS1 did not show significant interactions with p50 NF-κB and c-rel (Fig. 3.21*A*) making them available for nuclear translocation (Fig. 3.8*C* and Fig. 3.21*B*) and activate c-rel/p50-dependent genes like IL-12. Therefore, it appears that NAC/GSH-OEt specifically activates SOCS1 that prevents p65 NF-κB to translocation to the nucleus without affecting c-rel and p50 NF-κB. As a result, p65-dependent TNF-α and iNOS genes (Khan et al., 2007; Mukhopadhyay et al., 1999; Mukhopadhyay et al., 2004; Ziegler-Heitbrock et al., 1993) were not induced in these macrophages whereas c-rel/c-rel- or c-rel/p50-dependant genes like IL-12 (Rahim et al., 2005) were induced.

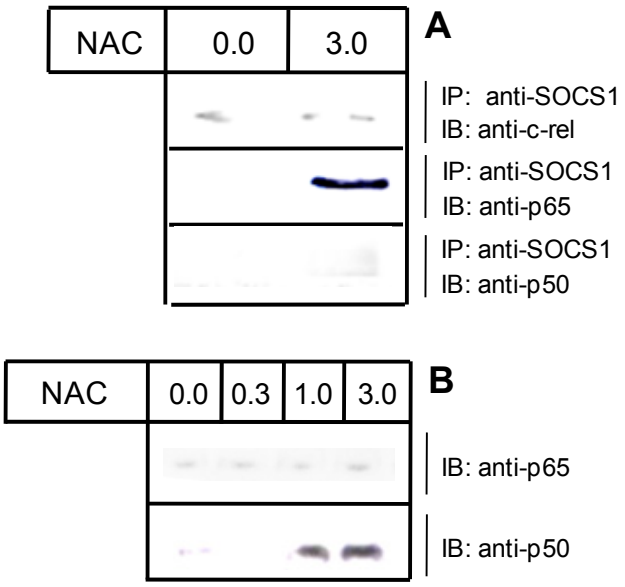

Figure 3.21. NAC targets the SOCS1 to regulate nuclear translocation of p65 NF-κB in RAW 264.7 macrophages. (*A*), The RAW 264.7 macrophages were either left untreated or treated with 3 mM NAC for 1 h. The whole cell extracts were prepared. The cell lysates were immunoprecipitated (IP) with goat anti-SOCS1 Ab, and the precipitates were immunoblotted (IB) with rabbit anti-p50 or anti-p65 or anti-c-rel Ab. (*B),* The p50 and p65 levels were measured in the nuclear extracts prepared from macrophages either left untreated or treated with NAC (0.3, 1 and 3 mM) for 1 h by Western blotting using anti-p65 or anti-p50 Ab. Data are representative of 5 independent experiments.

The results presented in Figs. 3.19, 3.20 and 3.21 indicate that SOCS1 is involved in the inhibition of nuclear p65 NF-κB in macrophages treated with 3 mM NAC. The role of SOCS1 in the regulation of nuclear p65 NF-κB and the p65 NF-κB-dependent genes like TNF-α and iNOS/NO in NAC (3 mM)-treated macrophages was further confirmed by gene silencing experiments using SOCS1-specific siRNA. The RAW 264.7 macrophages were transfected with control-siRNA (RAW-control group) or SOCS1-specific siRNA (RAW-SOCS1i group) and after 24 h, the macrophages were treated with 3 mM NAC. Cells were harvested after 1 h and depletion of SOCS1 by siRNA was assessed at mRNA by semi-quantitative RT-PCR (Fig. 3.22). The nuclear p65 level in these groups was measured by Western blotting using anti-p65 Ab. The RAW-control group and RAW-SOCS1i group were also used to investigate production of TNF-α and NO after treatment with 3 mM NAC. It could be observed that depletion of SOCS1 by SOCS1-specific siRNA (Fig. 3.22) resulted in an increased nuclear p65 level in the RAW-SOCS1i group as compared to RAW-control group when treated with 3 mM NAC (Fig. 3.23, compare lane 4 with lane 2).

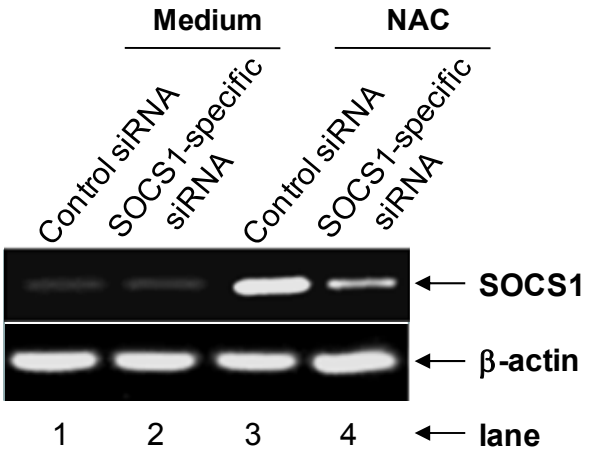

Figure 3.22. Specific knockdown of endogenous SOCS1 by siRNA. The RAW 264.7 were transfected with control-siRNA or SOCS1-specific siRNA following the method as described in the 'Materials and Methods'. After 24 h, cells were treated with medium or 3 mM NAC and harvested after 1 h for measuring SOCS1 mRNA levels by semi-quantitative RT-PCR. Data are representative of 3 independent experiments.

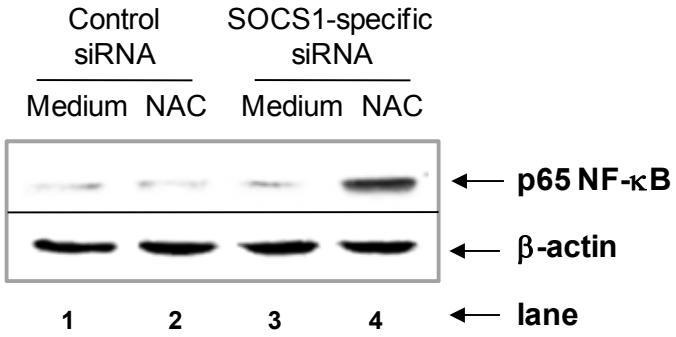

Figure 3.23. Specific knockdown of endogenous SOCS1 by siRNA increases nuclear translocation of p65 NF-κB in RAW 264.7 macrophages treated with 3 mM NAC. The RAW 264.7 macrophages were transfected with control-siRNA or SOCS1-specific siRNA. After 24 h, cells were treated with medium or 3 mM NAC and harvested after 1 h for measuring nuclear p65 levels by Western blotting using anti-p65 Ab. Data are representative of 3 independent experiments.

Since 3 mM NAC was found to increase nuclear p65 NF-κB (Fig. 3.23) in the absence of SOCS1, an increase in the production of p65 NF-κB-dependent genes like TNF-α and iNOS/NO was expected in this group. Therefore, the culture supernatants were harvested and the production of TNF-α as well as NO was observed in both RAW-SOCS1i and RAW-control groups treated with 3 mM NAC. Silencing of SOCS1 in RAW 264.7 macrophages enhanced production of both TNF-α as well as NO when RAW 264.7 macrophages were treated with 3 mM NAC (Figs. 3.24*A* and 3.24*B*, compare bar 4 with bar 2). Collectively, these results indicate that SOCS1 induction is increased in RAW 264.7 macrophages treated with 3 mM NAC, which prevents nuclear translocation of IκBα-released p65 NF-κB but not p50 or c-rel. This explains the possible mechanisms by which 3 mM NAC up-regulates IL-12 but not TNF-α or NO production in these macrophages.

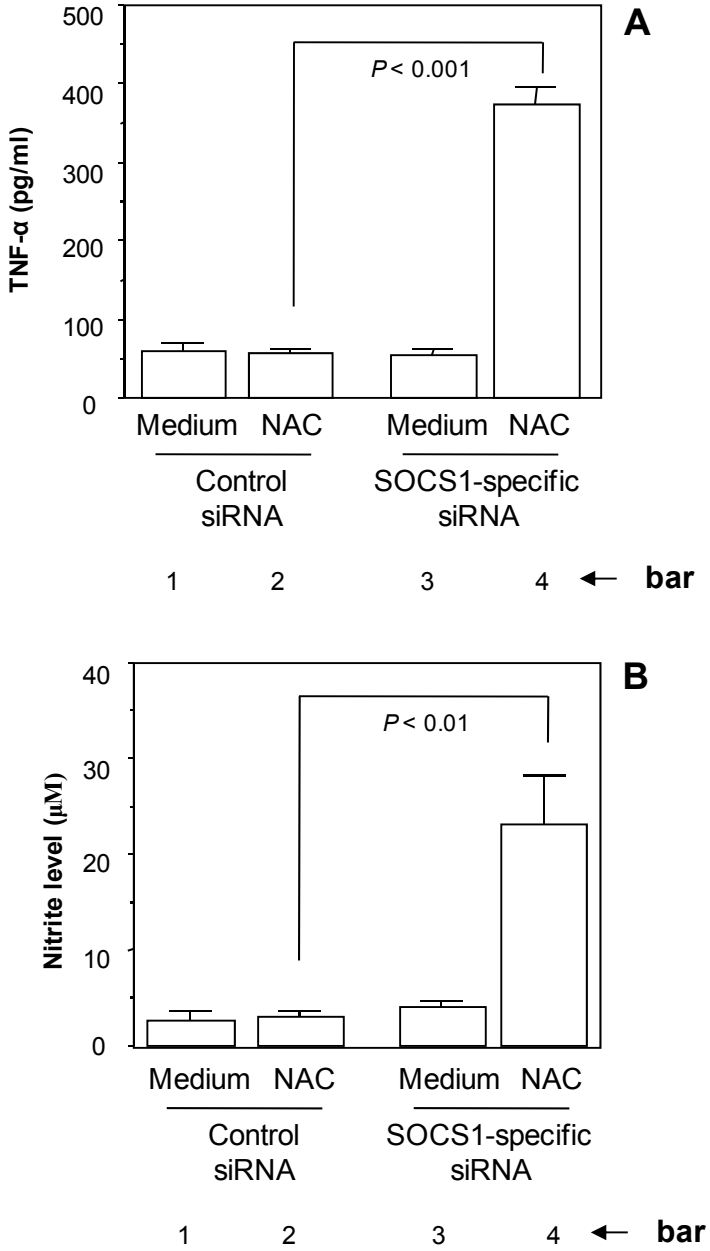

Figure 3.24. Suppression of SOCS1 by siRNA increases production of TNF-α and NO in RAW 264.7 macrophages treated with 3 mM NAC. The RAW 264.7 macrophages were transiently transfected with control siRNA or SOCS1-specific siRNA and after 24 h, cells were treated with medium alone or with 3 mM NAC and cultured for 48 h to measure production of TNF-α (mean ± SD) by EIA (*A*) and NO (mean ± SD) by Griess reaction (*B*). Data are representative of 3 independent experiments. *P* value was calculated by Student's *t* test.

3.6. Cell proliferation and IFN-γ response of PBMCs from TB patients to PPD is increased by 3 mM NAC

It has been reported that the Th1 T cell responses in TB patients with active infection are suppressed when purified protein derivative (PPD) was used as test antigen (Baliko et al., Khan et al., 2007). The suppressed Th1 response is known to favor intracellular survival of the *M. tuberculosis* bacilli (Baliko et al., 1998; Khan et al., 2008). The results in the present study indicate that NAC at 3 mM concentration potently increased IL-12 induction in macrophages. IL-12 is known to favor the Th1 response. Therefore, it was assumed that NAC at 3 mM concentration might act as Th1-adjuvant and activate Th1 response in TB patients. The IFN-γ production was measured as an indicator of Th1 and IL-5 as Th2. Therefore, it was examined whether the Th1 response to PPD was increased by NAC in PBMC cultures obtained from active TB patients. The PBMC were harvested from TB patients (n = 24) and cultured *in vitro* with PPD in the absence or presence of 3 mM NAC. After 96 h the culture supernatants were harvested for estimation of IFN-γ (a Th1 cytokine) and IL-5 (a Th2 cytokine) production by EIA. The T cell proliferation was measured by MTT assay. It could be observed that NAC at 3 mM concentration increased T cell proliferation in response to PPD (Fig. 3.25*A*) with increased IFN-γ (Fig. 3.25*B*) and decreased IL-5 (Fig. 3.25*C*) production.

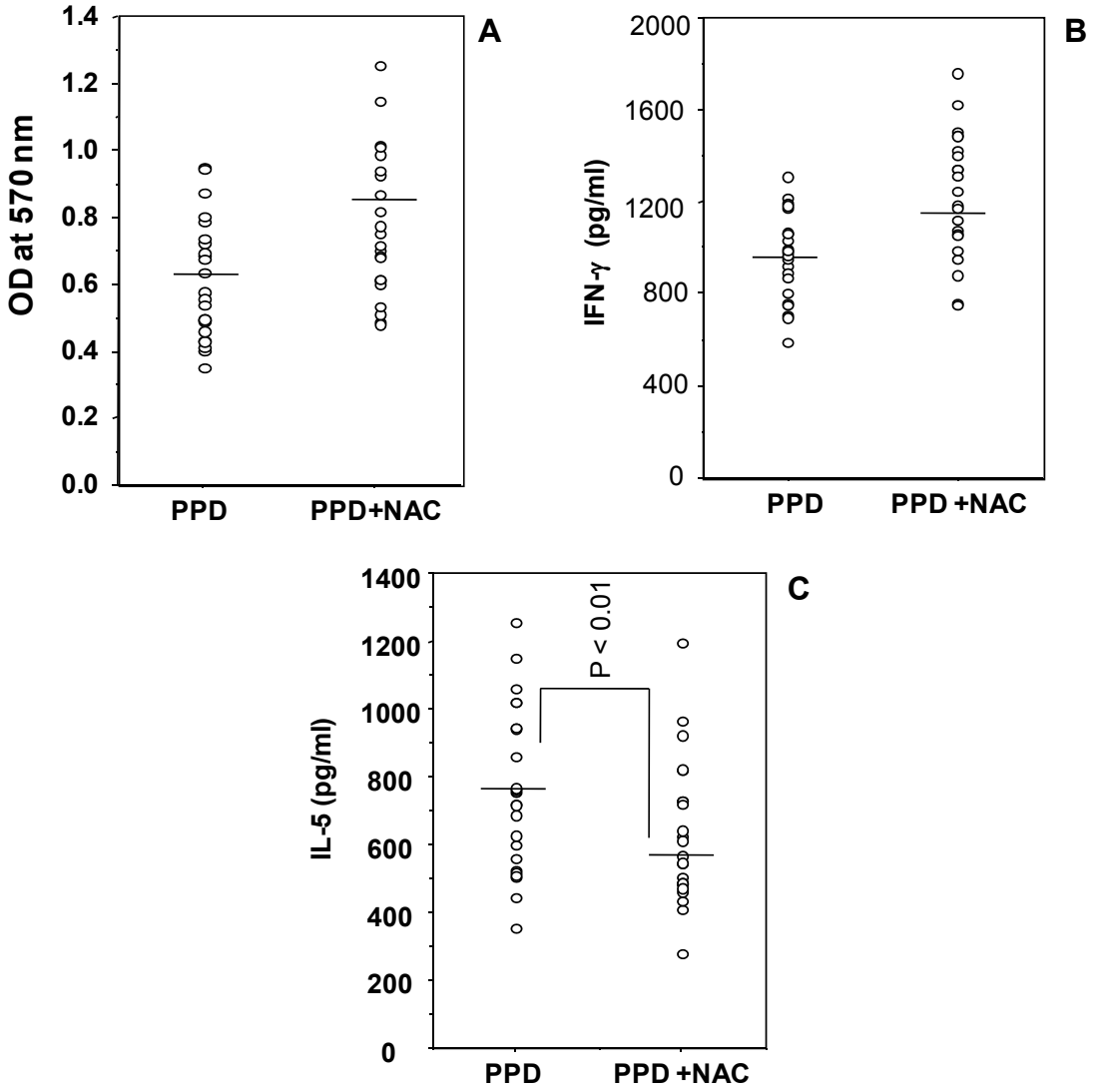

Figure 3.25. Cell proliferation and IFN-γ and IL-5 responses of PBMCs from TB patients to PPD is increased by 3 mM NAC. PBMCs (3 x 10^5/well) harvested from TB patients (n = 24) were activated with PPD (10 μg/ml) in the absence or presence of 3 mM NAC. After 4 days, cell proliferation was measured by MTT assay (*A*). The induction of IFN-γ (*B*) and IL-5 (*C*) in the culture supernatants of all the patients was measured by EIA. Each circle represents individual patient. *P* value was calculated by Student's *t* test.

3.7 Anti-BCG Th1 response in PBMCs from active TB patients is increased by 3 mM NAC

As NAC at 3 mM concentration increased anti-PPD Th1 response, it was expected that NAC can act as Th1 adjuvant to set up improved anti-BCG Th1 T cell immune response. PBMCs from 33 TB patients were incubated with *M. bovis* BCG in the absence or presence of 3 mM NAC. After 96 h, culture supernatants were harvested to measure the levels of IFN-γ and IL-5 by EIA and T cell proliferation by MTT assay. It was observed that NAC increased T cell proliferation to BCG (Fig. 3.26*A*) with a tendency of increased IFN-γ (Fig. 3.26*B*) and decreased IL-5 (Fig. 3.26*C*) production.

To critically assess possible role of the macrophages in the activation of Th1 immune response by 3 mM NAC, we isolated macrophages from individual TB patient and incubated for 2 h with medium or 3 mM NAC. The cells were washed to remove any external NAC and used as APCs. The purified T-cells were cultured with autologous macrophages (as APCs) pre-treated with medium or NAC (3 mM) along with BCG. After 96 h, culture supernatants were harvested to measure the levels of IL-5 and IFN-γ cytokines. The data revealed that the anti-BCG T cell response was increased (Fig. 3.27*A*) and was skewed towards the Th1-type as indicated by higher production of IFN-γ (Fig. 3.27*B*) and decreased production of IL-5 (Fig. 3.27*C*) in the groups that received NAC-

treated macrophages as APCs as compared to the groups receiving medium-treated macrophages as APCs. This indicates a critical role of macrophages in the NAC-mediated activation of anti-BCG Th1 response in TB patients. These results together demonstrate that NAC can be used as an efficient immunoadjuvant to activate Th1 response in TB patients.

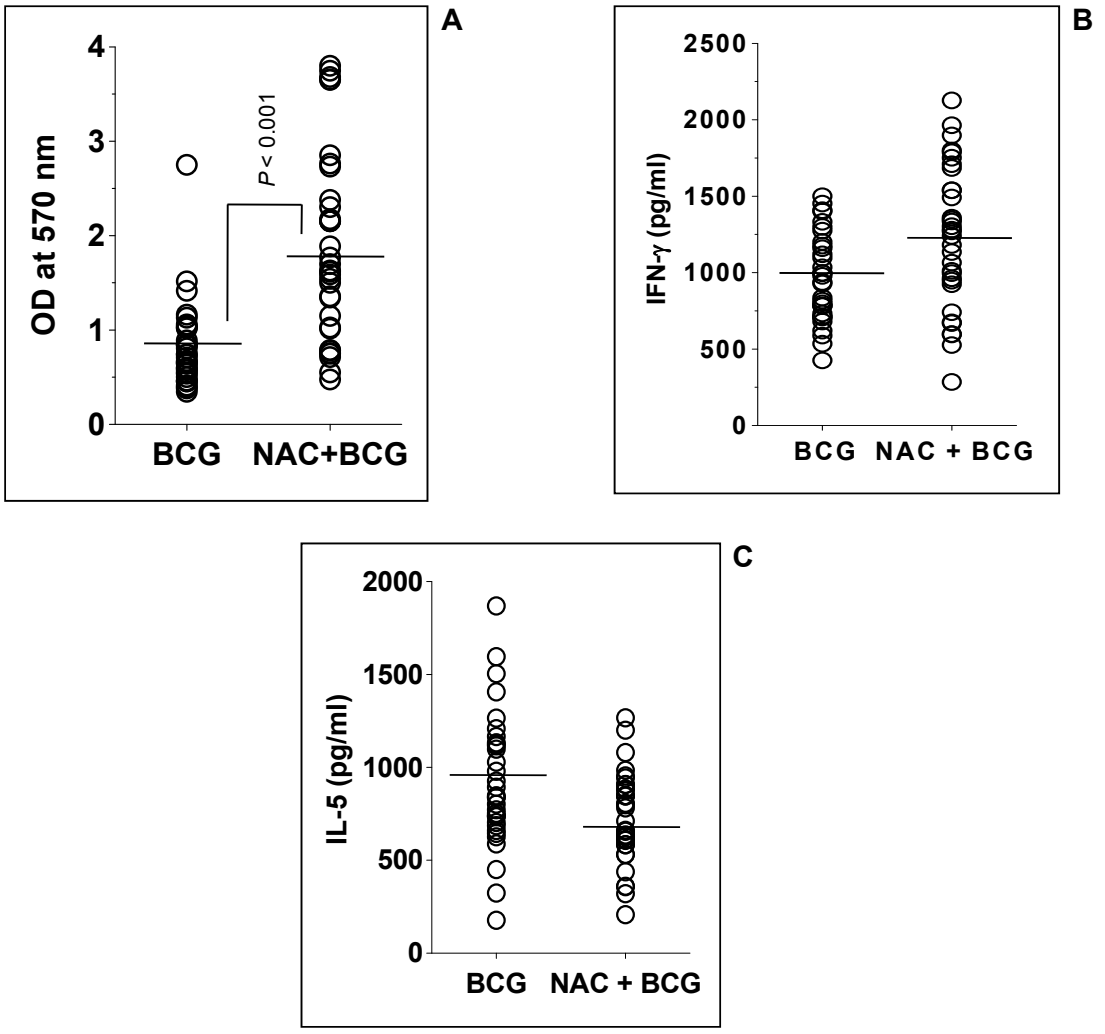

Figure 3.26. Cell proliferation and IFN-γ and IL-5 responses of PBMCs from TB patients to BCG is increased by 3 mM NAC. PBMCs (3 x 10^5/well) harvested from TB patients (n = 33) were activated with BCG (1 x 10^5/well) in the absence or presence of 3 mM NAC. After 4 days, cell proliferation was measured by MTT assay (*A*). The induction of IFN-γ (*B*) and IL-5 (*C*) in the culture supernatants of all the patients was measured by EIA. Each circle represents individual patient. *P* value was calculated by Student's *t* test.

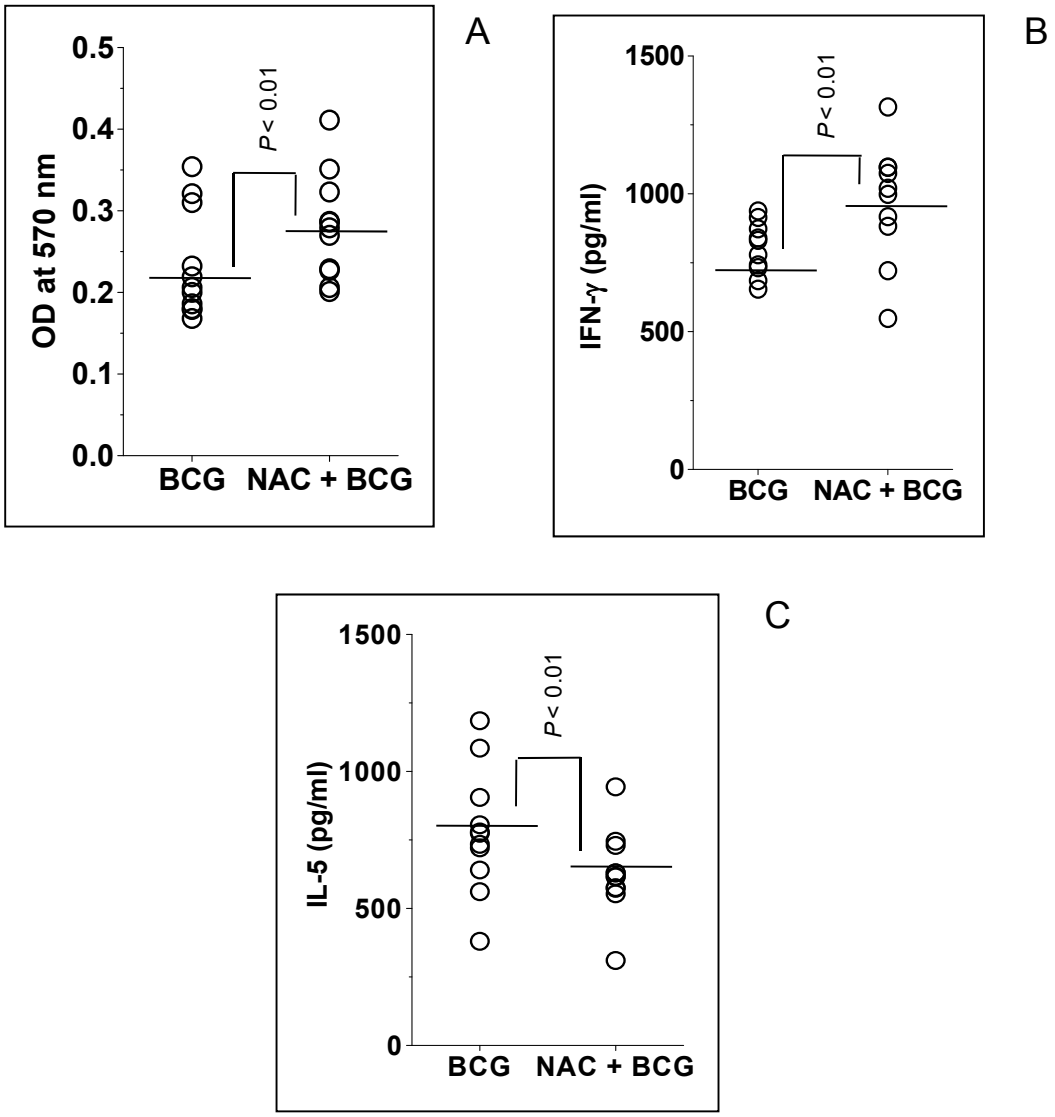

Figure 3.27. Anti-BCG T-cell responses are skewed to Th1-type when the NAC-treated macrophages (PBMC-derived) are used as APCs in T cell proliferation assay. The macrophages were purified from individual TB patient (n = 10) and pre-treated with 3 mM NAC. Cells were washed and cultured (1 x 10^5/well) with autologous nylon wool-purified T cells (3 x 10^5/well) in the presence of BCG (1 x 10^5/well). After 4 days, culture supernatants were harvested and cell proliferation was measured by MTT assay (*A*). The levels of IFN-γ (*B*) and IL-5 (*C*) in various culture supernatants were measured by EIA. Each circle represents individual patient. *P* value was calculated by Student's *t* test.

3.8 The anti-BCG T cell response phenotype is biased towards the Th1-type in mice immunized with BCG under the NAC cover

To further confirm a role of NAC to act as a Th1 immunoadjuvant to increase anti-BCG Th1 response, BALB/c mice were injected with BCG under the NAC cover as described in 'Materials and Methods'. Control groups received PBS alone. In an *in vitro* recall assay, when splenic cells were cultured with BCG (3×10^5), an increase in the magnitude of the anti-BCG T cell proliferative response was observed if immunization had been done under NAC cover (Fig. 3.28*A*). The cytokine balance (IFN-γ/IL-10 ratio) in NAC-treated mice shifted significantly in favor of Th1 responses (Fig. 3.28*D*), as indicated by increased IFN-γ production (Fig. 3.28*B*). The IL-10 levels were found to be equivalent in both the groups (Fig. 3.28C).

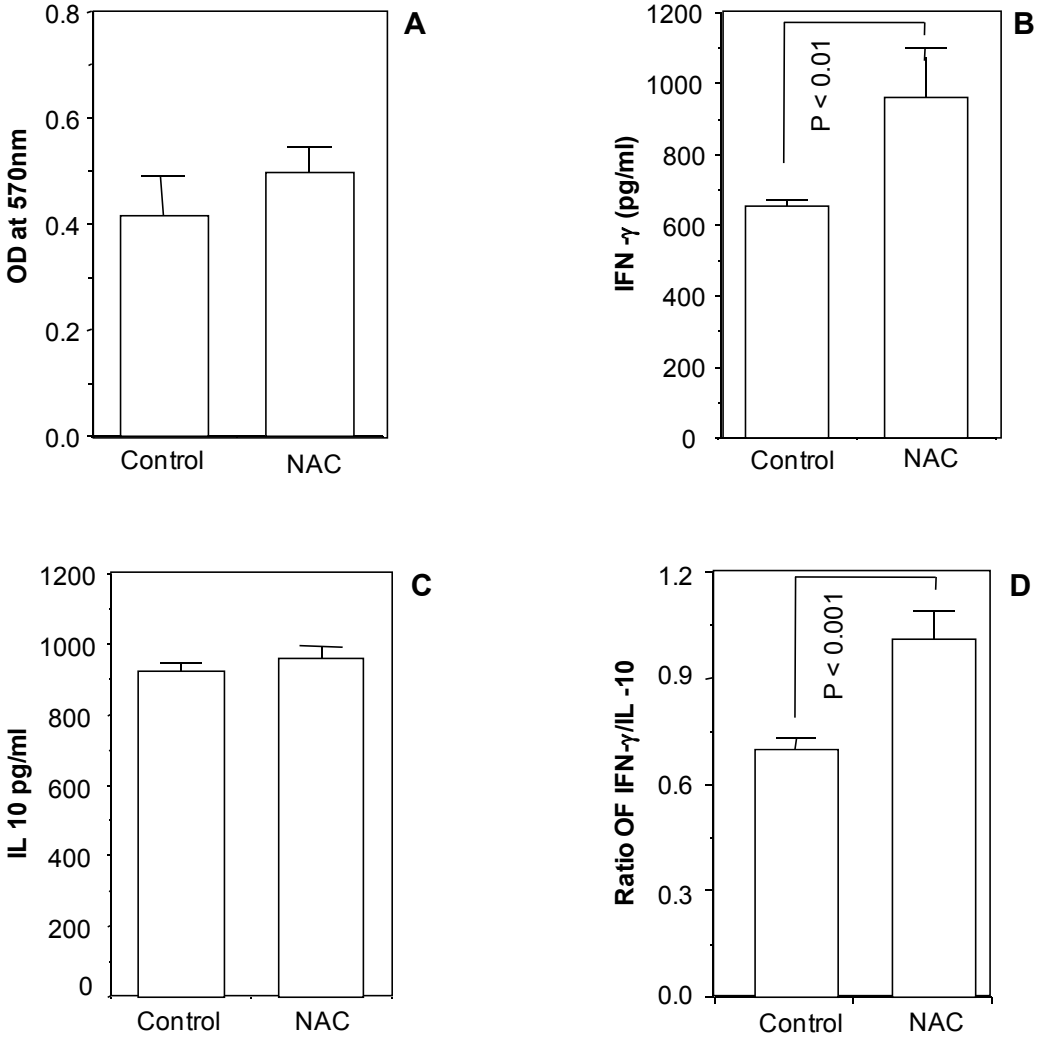

Figure 3.28. Anti-BCG T cell response in mice immunized with BCG under the NAC cover is biased towards the Th1-typ. Anti-BCG T cell responses of spleen cells from mice immunized with BCG in the absence or presence of NAC were measured at day 10 post-immunization. The magnitude of proliferative responses of spleen cells to BCG (3 X 10^5) is shown (*A*). The levels of IFN-γ (*B*) and IL-10 (*C*) observed in the culture supernatants were quantitated by EIA. The Th1/Th2 balances as represented by IFN-γ/IL-10 ratios are shown in (*D*). *P* value was calculated by Student's *t* test.

Chapter 4 - Discussion

Intracellular glutathione homeostasis plays a major role in the maintenance of intracellular redox environment and regulates several important cellular functions (Haddad and Harb, 2005). Growing evidences suggest that an altered cellular redox has a profound role in inflammation through the regulation of various kinases and redox-sensitive transcription factors such as NF-κB rel proteins, which differentially regulate the genes encoding various proinflammatory cytokines (Haddad and Harb, 2005; Kabe et al., 2005).

In the present study, the basal GSH/GSSG ratio in the unstimulated RAW 264.7 macrophages was found to be 200. Although there are some reports where the basal GSH/GSSG ratio is in the range of 3 to 10, many studies that used the recycling assay methods involving the reaction of 5,5'-dithio-bis(2-nitrobenzoic acid) and glutathione reductase indicate that the basal GSH/GSSG ratio in unstimulated RAW 264.7 macrophages can vary from more than 60 (Whitekus et al., 2002; Xiao et al., 2003) to more than 100 (Huh et al., 2006). Therefore, it appears that the basal GSH/GSSG ratio in unstimulated RAW264.7 macrophages can vary from experiment to experiment which could be due to differences in methods of sample processing as well as other factors like medium, FBS, glutathione reductase enzyme etc used in the assay.

The mechanisms by which the reduced and oxidized glutathione balance regulates the induction of various cytokines in unstimulated macrophages are not well understood. In the present study, it was demonstrated that the GSH/GSSG redox balance could modulate the IκBα signaling and the levels of CaM expression in macrophages which subsequently influenced the nuclear c-rel translocation and thereby regulated the levels of IL-12 in unstimulated macrophages. Although, some previous studies report that in LPS-activated macrophages, IL-12 p40 expression can be regulated by GSH ethyl esters by involving the JNK pathway (Utsugi et al., 2003; Utsugi et al., 2002), here it was observed that glutathione-redox-mediated regulation of IL-12 in unstimulated macrophages involved mainly the p38 MAPK and the CaM protein.

To study the effect of glutathione-redox balance on macrophages innate response, different concentrations of NAC was used to alter the intracellular glutathione-redox balance in unstimulated macrophages. NAC is a widely used cell permeable anti-oxidant that is converted into GSH by GCS (Mukherjee et al., 2007). It was observed that NAC, at lower concentrations (upto 3 mM) steadily increased the GSH levels without significantly affecting its conversion to its oxidized form, GSSG. However, when NAC was used at higher concentrations (10-20 mM), there was a significant conversion of GSH to GSSG, resulting in low GSH/GSSG ratio. Therefore, it appears that in unstimulated macrophages, NAC at low concentrations acts as an anti-oxidant and at higher concentrations it turns out to be a pro-oxidant. Interestingly, such concentration-dependant role reversal is not rare for NAC. In TNF-α-stimulated endothelial cells, NAC

could attenuate TNF-α-induced intercellular adhesion molecule-1 (ICAM-1) expression at low concentrations, but at higher concentrations, ICAM-1 expression was enhanced (Mukherjee et al., 2007). Interestingly, in several *in vivo* studies, high dose of NAC was found to act as a pro-oxidant rather than an anti-oxidant. High doses of NAC were found to be pro-oxidative in function in healthy rat striatum (Harvey et al., 2008). Similarly, a low dose of NAC protected rats against endotoxin-mediated oxidative stress whereas a high dose increased their mortality presumably because of its pro-oxidant action (Sprong et al., 1998). NAC at high dose was also found to act as a pro-oxidant as it could decrease the GSH levels and increase the GSSG levels in healthy human subjects (Kleinveld et al., 1992). It will be pertinent to mention here that, NAC can also act as pro-oxidant in the presence of certain compounds like vitamin B12b (Solov'eva et al., 2007). Nevertheless, NAC can act as pro-oxidant or anti-oxidant depending on the pre-existing redox state. Pre-treatment of NAC was found to counteract the ethanol-induced oxidative stress in liver, however, on the contrary, post-treatment was found to aggravate the liver injury as NAC behaved as a pro-oxidant in such circumstances (Wang et al., 2006). Similarly, pre-treatment of NAC was able to protect LPS-induced intra-uterine fetal death and growth retardation, where as post-treatment aggravated these situations as a pro-oxidant (Xu et al., 2005). Therefore, it appears that whether NAC may act as a pro-oxidant or anti-oxidant depends on some factors like concentration and the existing redox status. As the unstimulated cells are used in these experiments, which already have an existing pool of GSH, addition of NAC at high concentration is likely to shoot the cellular GSH levels abruptly high leading to production of some kind of cellular

stress that probably rapidly converted part of the large GSH pool into GSSG and thus skewed the GSH/GSSG ratio toward a pro-oxidant state. Although, NAC is not known to change the expression levels of genes involved in the glutathione recycling, but is known to alter the activities of these enzymes in TNF-α-stimulated endothelial cells (Mukherjee et al., 2007). It may be possible that the activities of these enzymes are differently altered by different concentrations of NAC in unstimulated cells leading to such biphasic activities of NAC.

When the effect of glutathione-redox on the production of macrophage innate cytokines in unstimulated macrophages was examined, it was observed that a high GSH/GSSG, induced by lower concentrations of NAC, significantly increased IL-12 production in both RAW 264.7 and monocyte derived macrophages. However, at higher concentrations, IL-12 production is significantly inhibited and NAC did not have any apparent cytotoxic effect at these higher concentrations used. Interestingly, the levels of TNF-α did not significantly change in all the concentrations of NAC used. The concentration-dependent regulation of IL-12 by NAC was found to be due to differential nuclear translocation of c-rel, which is known to be critical for IL-12 transcription (Rahim et al., 2005), in spite of increased IκBα phosphorylation in all the concentrations of NAC used. The differential nuclear c-rel levels could be attributed to an increased level of CaM protein only at higher concentrations of NAC. Recently it has been shown that CaM can interact and regulate the activities of IκBα-released NF-κB family members by differentially regulating nuclear localization of c-rel and p65 as CaM-bound p65 could

localize into the nucleus, whereas CaM-bound c-rel is sequestered and retained in the cytoplasm (Antonsson et al., 2003). An oxidized glutathione rich environment imparted by treating the macrophages with high concentrations of NAC could result in an increased level of cytoplasmic CaM protein, which in turn sequesters the c-rel to prevent its nuclear translocation. A link between the CaM expression and IL-12 p40 regulation has already been demonstrated (Khan et al., 2006). In this study for the first time it is shown that a changing GSH/GSSG balance can alter the levels of CaM protein and thereby affect the levels of IL-12 p40 in native macrophages. The CaM protein was found to be regulated by the redox-balance through the p38 MAPK as was indicated earlier (Boddupalli et al., 2007).

Interestingly, although there was increased IκBα phosphorylation in all the concentrations of NAC used, the levels of TNF-α remained largely unchanged. The c-rel as well as the other NF-κB factors like p50 and p65 are known to be sequestered by the IκBα in the cytoplasm and are released to translocate to nucleus upon phosphorylation of IκBα (Hoffmann et al., 2006; Karin and Ben-Neriah, 2000; May and Ghosh, 1997). Therefore, an increase in the nuclear levels of p50 and p65 NF-κB factors along with c-rel was expected at 3 mM NAC concentration. However, NAC at 3 mM concentration could increase the nuclear levels of p50 and c-rel but the p65 levels were barely detectable under identical conditions. This causes a decrease in a TNF-α induction, whose transcription is largely controlled by p65 NF-κB (Khan et al., 2007; Ziegler-Heitbrock et al., 1993). Since, the SOCS1 is known to negatively regulate NF-κB activation by specifically targeting the p65 through proteasome-mediated degradation

(Ryo et al., 2003), a direct role of SOCS1 in NAC-mediated inhibition of nuclear translocation of p65 was speculated. As NAC treatment (3 mM) augmented IL-12 synthesis but not TNF-α, it was assumed that NAC probably induces SOCS1 which in turn negatively affects nuclear translocation of p65 by targeting it to the proteasome (Ryo et al., 2003). When the levels of SOCS1 were examined in macrophages treated with 3 mM concentration of NAC, an increase in SOCS1 expression was observed at 3 mM NAC concentration. Similar changes in glutathione-redox balance in macrophages by 5 mM GSH-OEt also increased SOCS1 expression indicating that the reduced glutathione environment increases SOCS1 expression in macrophages. The SOCS1 was found to strongly interact with the p65 subunit of NF-κB preventing its nuclear translocation in macrophages treated with 3 mM NAC. As a result, p65-dependent TNF-α and the iNOS genes (Ziegler-Heitbrock et al., 1993, Khan et al., 2007, 19:477-486; Mukhopadhyay et al., 1999, Mukhopadhyay et al., 2004) were not induced whereas c-rel/c-rel- or c-rel/p50-dependant genes like IL-12 (Rahim et al., 2005) were activated. The role of SOCS1 in the inhibition of nuclear p65 as well as TNF-α and iNOS/NO expression was further confirmed by gene silencing experiments using SOCS1-specific siRNA. It was observed that silencing of SOCS1 in RAW 264.7 macrophages enhanced both the nuclear p65 level and production of TNF-α as well as NO in the presence of 3 mM NAC. Thus, the glutathione-redox specifically attenuates p65-dependent activation of TNF-α and iNOS/NO in macrophage through specific activation of SOCS1. Therefore, glutathione-redox appears to play a pivotal role in regulating macrophage signaling involving the CaM and SOCS1 to differentially regulate various NF-κB family-dependent cytokines.

There has been intense debate regarding the exact effect of NAC on the NF-κB activity. It is reported that 20 mM NAC downregulates basal NF-κB DNA binding in RAW 264.7 macrophages and also inhibits LPS-stimulated NF-κB DNA binding in these cells (Song et al., 2004). In contrast, few studies indicate that NAC fails to inhibit LPS-induced activation of NF-κB in RAW 264.7 macrophages (Wadsworth and Koop, 1999), yet in other studies, NAC has been shown to inhibit activation of NF-κB in alveolar macrophages induced by TNF-α (Li et al., 2006). A role of IκB kinase α (IKKα) is also proposed in the NAC-mediated regulation of NF-κB signaling in TNF-α-stimulated endothelial cells (Mukherjee et al., 2007). These apparent discrepancies in the role of NAC in regulating NF-κB signaling could be explained by the fact that NAC behaves differently when used at different concentrations as well as differences in redox status. Also, NAC may specifically induce different sets of genes depending on the type and redox status of the cell. The complex interactions of these gene products probably dictate the course of NF-κB-mediated signaling whether to be activated or inhibited. In fact the data in the present study, at least partly explain how NAC can differentially regulate the nuclear translocation of different members of the NF-κB family and therefore promote transcription of IL-12 and TNF-α differently. A detailed study in the upstream signaling cascades may be helpful for better understanding the differential effect of NAC on NF-κB-dependent signaling in macrophages activated with various stimulators.

Results in the present study underscore the critical importance of glutathione-redox status in regulating induction of innate cytokines in macrophages. This can interestingly constitute a point of hijacking the host immune response by the intracellular macrophage pathogens like *M. tuberculosis*. Interestingly, studies also indicate that TB patients have an altered glutathione status (Venketaraman et al., 2008). GSH depletion can also lead to suppression of Th1 cytokine profile and skew the T cell response biased towards the Th2 type (Peterson et al., 1998). Therefore, it is possible that the *M. tuberculosis* has the ability to modulate the GSH/GSSG levels in macrophages to polarize the immune environment to its favor. One of the immune-effectors regulated by GSH/GSSG balance is found to be the IL-12 cytokine as described in this study. IL-12 is known to activate Th1 T-cell immune response (Trinchieri, 2003) and IFN-γ production which are crucial for inducing protection against the pathogenic mycobacteria (Stewart et al., 2003). Indeed, IL-12 was shown to be a particularly potent adjunct for chemotherapy of *M. avium* infection in immunocompetent mice and resulted in more effective control of the pathogen without the need for increased drug dosage. The role of IL-12 in anti-mycobacterial protective immunity has been confirmed using the IL-12 knock-out mice. The C57Bl/6 IL-12 knock-out mice were found to be more susceptible as compared to the wild type mice to bacillus Calmette-Guerin bacteria. These mice revealed increased bacterial load which was associated with poorer granuloma formation and the leukocyte recruitment to the site of infection and also reduced secretion of both IFN-γ and TNF-α (Wakeham et al., 1998). The important role of IL-12 for T-cell mediated immunity to mycobacterial infection is demonstrated further in

patients with defective IL-12 receptors on T-cells (Modlin and Barnes, 1995). These patients were poor in producing IFN-γ by both the T cells and natural killer (NK) cells. The IL-12 also contributes to the resistance by inducing the production of chemokines that attract macrophages to the site of infection. These cells quarantine the inside a granulomatous lesion called tubercle. The massive activation of the macrophages that occurs within the tubercle often results in the concentrated release of the lytic enzymes which are responsible to destroy the infected as well as the nearby healthy cells resulting in circular lesion of necrotic tissue (Flynn et al., 1995). Thus it is possible that the bacilli inhibit IL-12 production and Th1 response (Russell et al., 2009) by targeting the glutahthione-redox signaling of the macrophages. Therefore, it is important to increase IL-12 production and Th1 T cell immune response to induce effective antimycobacterial immunity. In this context, the observation that NAC could increase IL-12 induction in macrophages and also help in Th1 polarization suggest that NAC can be used as an immunoadjuvant to improve immune response in patients with active TB infection. In fact, NAC was found to act as an immunoenhancer that modulate Th1/Th2 balance in HIV+ CD4+ T-cells (Eylar et al., 1995).

Studies has shown that BCG vaccine failed to have significant protective efficiency against tuberculosis, so alternatives approaches such as adjuncts to existing microbial therapies are needed to control the tuberculosis epidemic. Furthermore, various clinical evidences have suggest that enhancement of the host's immune responses is critical for increasing the efficacy of antimicrobials in immunocompromised patients as well as in

patients whose responses to antimicrobials is inadequate and also for enhancing the immunogenecity of BCG and other anti-TB vaccine(s). In this context, results in the present study demonstrate that NAC when used at 3 mM concentration could improve anti-BCG Th1 response in PBMC cultures obtained from active TB patients. Immunization of BALB/c mice with BCG under the NAC cover was found to result in increased T cell proliferation and a predominant Th1 T cell response against BCG as indicated by an increase in the IFN-γ production. Therefore, NAC-mediated manipulation of intracellular glutathione balance can be used to devise immune-therapeutics to improve the anti-BCG Th1 response in TB patients. This strategy can also be used in cancer treatment to improve Th1 immune response (Luo et al., 1999). This study also provides strategies to pharmacologically regulate IL-12 induction by manipulating GSH and GSSG levels in macrophages. However, it should be used with caution for immunotherapeutic applications as higher concentrations may lead to development of undesirable and opposite effects.

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Glutathione-redox balance regulates c-rel driven IL-12 production in macrophages: Possible implications in anti-tuberculosis immunotherapy.

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The redox status of the cells can regulate the innate immunity presumably by acting as signaling regulator that can control a number of immunoregulatory genes. Glutathione-redox environment is crucial and critical for various important cellular functions. The cellular glutathione-redox environment can alter protein functions by reversible disulfide bond formation on various proteins including phosphatases, kinases and transcription factors. It is also known to protect cells against oxidative stress and regulate programmed cell death. However, the role of glutathione redox balance in regulating macrophage innate signaling is not well understood. In this study, alteration in the intracellular glutathione-redox balance in macrophages by N-acetyl-L-cystein (NAC) or glutathione ethyl ester reduced (GSH-OEt) was found to modulate innate-effector functions of macrophages. The intracellular glutathione-redox environment of macrophages was found to be critically dependent on the concentration of NAC used. A predominantly reducing environment induced in macrophages by treating with lower concentration of NAC (upto 3 mM) was found to increase the production of IL-12 without affecting IL-10 and TNF-α production. Similarly change in glutathione redox balance by a cell permeable GSH-OEt had similar affect on IL-12 induction as observed in NAC. At 3 mM concentration, NAC was found to enhance phophorylation and degradation of IκBα resulting in increased nuclear translocation of c-rel and p50 NF-κB. However, nuclear p65 NF-κB was found to be poorly expressed which was presumably due to increased expression of SOCS1 at 3mM concentration of NAC used. SOCS1 expression is also increased when macrophages were treated with 5 mM GSH-OEt. It was observed that SOCS1 could negatively affect nuclear translocation of p65 by

targeting it to the proteasome (Ryo et al., 2003). However, SOCS1 did not interact with p50 NF-κB and c-rel, making them available for nuclear translocation that activated c-rel/c-rel or c-rel/p50-dependent genes like IL-12. This was further confirmed by gene silencing experiments using SOCS1-specific siRNA. Silencing of SOCS1 in RAW 264.7 macrophages using SOCS1-specific siRNA resulted in increased nuclear p65 as well as an enhanced production of both TNF-α and nitric oxide in the presence of 3 mM NAC. Thus, a reduced glutathione rich environment can specifically affect the IκBα-SOCS1 signaling, as a result of which p65-dependent TNF-α and the iNOS genes were not induced in these macrophages whereas c-rel/c-rel- or c-rel/p50-dependant genes like IL-12 were induced.

Interestingly at higher concentrations (10 mM and above), NAC as well as GSH-OEt increased the oxidized glutathione (GSSG) levels, making the overall intracellular redox environment more prooxidant in nature. At theses concentration, the IL-12 induction in macrophages was lower as compared to that of 3mM NAC. Although, at higher concentrations of NAC (10 and 20 mM), the IκBα phosphorylation was increased in macrophages, nuclear translocation of c-rel is inhibited which was due to increased expression of calmodulin (CaM) protein. The CaM was found to bind and sequester the IκBα-released c-rel in the cytoplasm and thereby preventing its translocation to the nucleus, resulting in the suppression of IL-12 p40 transcription. A pharmacological inhibitor of CaM activity, the TFP was found to increase both c-rel and IL-12 expression in macrophages, indicating a role of CAM in the IL-12 signaling by oxidized glutathione.

CaM was found to be regulated upstream by p38 MAPK in these macrophages. These results suggest that glutathione-redox balance can regulate intracellular signaling in native macrophages affecting the signal transduction of NF-κB/rel-family transcription factors. These in turn show a regulatory effect on the important effector functions of macrophages like induction of IL-12 and TNF-α cytokines as well as production of nitric oxide.

Since NAC at 3 mM concentration was found to increase IL-12 induction in macrophages and IL-12 is known to promote Th1 immune response, the immunotherapeutic potential of NAC in promoting Th1 response in tuberculosis was investigated. It could be observed that both anti-PPD and anti-BCG Th1 response was increased in PBMCs obtained from patients with active TB infection. When macrophages were isolated and treated with 3 mM NAC and cultured with autologous purified T cells, it was found that the anti-BCG T cell response was up-regulated and skewed towards the Th1-type as indicated by higher production of IFN-γ and decreased production of IL-5 in the groups that received NAC-treated macrophages as APCs as compared to the groups receiving medium-treated macrophages as APCs. This indicates critical role of macrophages in the NAC-mediated activation of Th1 response in tuberculosis. These results together demonstrate that NAC can be used as an efficient immunoadjuvant to activate Th1 response in TB patients. Again, the ability of NAC as a Th1 immunoadjuvant was investigated in mice model *in vivo* where it could increase anti-BCG Th1 response. There was an increase in T cell proliferation as well as a higher IFN-γ/IL-10 in mice immunized with BCG under NAC

cover as compared to the control mice that were immunized with BCG in presence of PBS alone.

In summary these studies indicate that intracellular glutathione-redox balance plays a critical role in regulating IL-12 induction in unstimulated macrophages and NAC can be used in tailoring the macrophages to induce an enhanced Th1 response that may be helpful in controlling tuberculosis and other pathophysiological disorders with a defective Th1 response.

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List of publications

1. **Alam,K.,** Ghousunnissa, S., Nair, S., Valluri VL and Sangita Mukhopadhyay
   Glutathione-redox balance regulates c-rel driven IL-12 production in macrophages: Possible implications in anti-tuberculosis immunotherapy.
   J Immunol. 2010 Mar 15;184(6):2918-29. Epub 2010 Feb 17.
2. Khan, N., **Alam, K.,** Mande, S.C., Valluri, V.L., Hasnain, S.E., Mukhopadhyay, S.
   Mycobacterium tuberculosis heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages.
   Cell Microbiol. 2008, Aug; 10 (8): 1711-22. Epub 2008 Apr 17.
3. Khan, N., **Alam, K.**, Nair, S., Valluri, V.L., Murthy, K.J., Mukhopadhyay, S.
   Association of strong immune responses to PPE protein Rv1168c with active tuberculosis.
   Clin Vaccine Immunol. 2008, Jun; 15(6):974-80. Epub 2008 Apr 9.
4. **Alam, K.,** Khan, N., Mukhopadhyay, S.
   Glutathione redox balance affect the amplitude and duration of Toll like receptor triggered downstream signaling by differentially regulating NF-κB and AP-1 transcription factors **(Manuscript under preparation)**

Glutathione-Redox Balance Regulates c-rel–Driven IL-12 Production in Macrophages: Possible Implications in Antituberculosis Immunotherapy

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The glutathione-redox balance, expressed as the ratio of intracellular reduced glutathione (GSH) and oxidized glutathione, plays an important role in regulating cellular immune responses. In the current study, we demonstrate that alteration of glutathione-redox balance in macrophages by GSH donors like cell-permeable glutathione ethyl ester reduced or *N*-acetyl-L-cysteine (NAC) can differentially regulate production of IL-12 cytokine in macrophages. A low concentration of NAC increased IL-12 p40/p70 production, whereas at high concentration, IL-12 production was inhibited due to increased calmodulin expression that binds and sequesters c-rel in the cytoplasm. Although NAC treatment increased the IκBα phosphorylation, it failed to increase TNF-α levels due to enhanced expression of suppressor of cytokine signaling 1, which specifically prevented nuclear translocation of p65 NF-κB. We demonstrate that NAC at 3 mM concentration could increase bacillus Calmette-Guérin–induced IFN-γ production by PBMCs from patients with active tuberculosis and shifts the anti–bacillus Calmette-Guérin immune response toward the protective Th1 type. Our results indicate that redox balance of glutathione plays a critical role in regulating IL-12 induction in native macrophages, and NAC can be used in tailoring macrophages to induce enhanced Th1 response that may be helpful to control tuberculosis and other pathophysiological disorders. ***The Journal of Immunology*****, 2010, 184: 2918–2929.**

Glutathione is the most predominant intracellular low m.w. thiol and acts as a reducing agent and an antioxidant. Glutathione is implicated in many cellular functions, including synthesis and degradation of protein and DNA. The intracellular redox environment is mainly controlled by the glutathione-redox, which is defined as the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) (1) and plays critical roles in maintaining cellular homeostasis and various physiological functions (2). The cellular glutathione-redox dynamically regulates protein functions by reversible disulfide bond formation on variety of proteins, including phosphatases, kinases, and transcription factors (3–5) and is known to protect against oxidative stress (6, 7). The glutathione-redox balance in macrophages is shown to be critical for mounting innate immune responses and has also been implicated in many of the pathological conditions (2, 8–10), indicating its possible potential use as a therapeutic agent. However, redox-based therapy is still in its early developmental phases.

It has been observed that triggering of macrophage receptors by pathogen products could change the macrophage redox status (9) and modulate the cytokine milieu (11, 12). Various studies indicate that glutathione levels in APCs can influence the Th response (13, 14). It is well known that the Th1/Th2 lineage commitment depends to a great extent on the cytokine milieu induced during innate activation of macrophages (15, 16). For example, IL-12 and TNF-α activate the Th1 T cell, whereas IL-10 cytokine favors the Th2 T cell development. Therefore, understanding the signaling pathways modulated by the glutathione-redox in macrophages and thereby influencing induction of these cytokines is important to understand how the T cell microenvironment is controlled during various pathophysiological conditions. However, the molecular mechanisms by which glutathione-redox can directly control macrophage functions and cytokine induction profile are largely not understood, other than in a few cases like GSH-mediated regulation of NF-κB (6, 7).

There are indications that Th1 immune response is downregulated in patients with active tuberculosis (TB) infection (17). Interestingly, studies also indicate that patients with TB have altered glutathione balance (18). However, it is not clear whether *Mycobacterium tuberculosis* modulates the T cell responses by altering the glutathione-redox balance in macrophages. In the current study, we demonstrate that IL-12 induction in native macrophages is controlled directly by the intracellular glutathione-redox through calmodulin (CaM) and c-rel, and manipulation of the macrophage-glutathione-redox can influence in vitro cellular immune response of PBMCs obtained from patients with active tuberculosis to bacillus Calmette-Guérin (BCG).

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Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; BSO, L-buthionine-(S,R)-sulphoximine; CaM, calmodulin; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DNp38, dominant-negative mutant of p38; EIA, enzyme immunoassay; GSH, reduced glutathione; GSH-OEt, glutathione ethyl ester reduced; GSSG, oxidized glutathione; HSD, honestly significant difference; IB, immunoblotting; ΔIκBα, phosphorylation-defective IκBα; iNOS, inducible NO synthase; IP, immunoprecipitation; MDM, monocyte-derived macrophage; NAC, *N*-acetyl-L-cysteine; pp38, phospho-p38; RAW-control, control-siRNA; RAW-SOCS1i, SOCS1-specific siRNA; ROS, reactive oxygen species; siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; TB, tuberculosis; TFP, trifluoperazine.

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Mycobacterium tuberculosis heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages

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Summary

The T-helper (Th) 1 T-cell response to purified protein derivative (PPD) is known to be suppressed in tuberculosis patients which favours intracellular survival of the bacilli. We demonstrate that the *Mycobacterium tuberculosis* heat shock protein 60 (Mtbhsp60) plays an important role to skew the anti-PPD T-cell response towards the Th2 type when macrophages were used as antigen presenting cells. We found that the PPD-induced IL-12 p40 was downregulated in macrophages by Mtbhsp60. The Mtbhsp60 preferentially induced Toll-like receptor (TLR) 2 without affecting TLR4 expression on macrophages. Interaction of Mtbhsp60 with TLR2 resulted in significant suppression of nuclear c-rel and consequently IL-12 p40 levels in PPD-activated macrophages. Our findings reveal a unique role of the Mtbhsp60 favouring development of Th2 type response by upregulating surface expression of TLR2 on macrophages which could be a survival strategy adopted by the bacilli.

Introduction

Interaction between the pathogen and the host is a dynamic confrontation where the microbe's strategy of survival by implementing various devices that challenges the formidable defences of the host immune system. A complex interplay between the 'host's defence mechanisms' and 'attempts to circumvent these defences by *Mycobacterium tuberculosis*' plays a key role in determining the outcome of tuberculosis (TB) infection.

Various studies reveal that *M. tuberculosis* promotes an environment characterized by T-helper (Th) 2 cytokines during infection (Hernandez-Pando *et al*., 1996; Rook *et al*., 2005). The observation that the anti-purified protein derivative (PPD) Th1 T-cell response is far less pronounced in TB patient (Baliko *et al*., 1998; Wilsher *et al*., 1999) suggests the possibility that an active TB infection is associated with a decrease in Th1 response. Therefore, it seems that failure to resolve infection in susceptible individuals could be a consequence of strategies used by the *M. tuberculosis* to suppress Th1 response. The interleukin (IL)-12 cytokine secreted by the antigen presenting cells (APCs) during the innate immune response (D'Andrea *et al*., 1992; Trinchieri, 1997) plays an important role in stimulating IFN-γ production and a Th1 T-cell response (Hsieh *et al*., 1993; Trinchieri, 1994; Magram *et al*., 1996; Mukhopadhyay *et al*., 1999). The bacilli being the macrophage-resident (Fenton, 1998; Schnappinger *et al*., 2003) are likely to suppress IL-12 induction in macrophages (Cooper *et al*., 1997; Fenton, 1998; Flynn and Chan, 2003) to create a favourable Th2 environment for it to thrive.

Heat shock proteins (hsps) were initially identified as prominent antigens in a range of infectious diseases and autoimmune disorders (Young *et al*., 1988; Zugel and Kaufmann, 1999). In addition to immune recognition of the protein themselves, the major functions of hsps are to act as molecular chaperones to assist in folding and assembly of polypeptides within the cell (Zugel and Kaufmann, 1999). Various studies reveal that the biochemical features of *M. tuberculosis* hsp60 (Mtbhsp60) deviate significantly from the characteristic properties of the *Escherichia coli* hsp60 (Gaston, 2002; Qamra *et al*., 2004). Interestingly, an attempt to monitor gene expression patterns of mycobacteria residing within macrophages revealed upregulation of Mtbhsp60 protein (Young and Garbe, 1991; Zugel and Kaufmann, 1999; Monahan *et al*., 2001), suggesting that the protein might offer a survival advantage to mycobacteria within the host (Shinnick, 1991).

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Association of Strong Immune Responses to PPE Protein Rv1168c with Active Tuberculosis▽

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Accurate diagnosis of tuberculosis (TB) infection is critical for the treatment, prevention, and control of TB. Conventional diagnostic tests based on purified protein derivative (PPD) do not achieve the required diagnostic sensitivity. Therefore, in this study, we have evaluated the immunogenic properties of Rv1168c, a member of the PPE family, in comparison with PPD, which is routinely used in the tuberculin test, and Hsp60 and ESAT-6, well-known immunodominant antigens of *Mycobacterium tuberculosis*. In a conventional enzyme immunoassay, the recombinant Rv1168c protein displayed stronger immunoreactivity against the sera obtained from patients with clinically active TB than did PPD, Hsp60, or ESAT-6 and could distinguish TB patients from *Mycobacterium bovis* BCG-vaccinated controls. Interestingly, Rv1168c antigen permits diagnosis of smear-negative pulmonary TB as well as extrapulmonary TB cases, which are often difficult to diagnose by conventional tests. The immunodominant nature of Rv1168c makes it a promising candidate to use in serodiagnosis of TB. In addition, our studies also show that Rv1168c is a potent T-cell antigen which elicits a strong gamma interferon response in sensitized peripheral blood mononuclear cells obtained from TB patients.

Tuberculosis (TB) remains a significant global public health concern and is a major cause of death in adults by a single bacterial agent (39). The increasing global health burden of tuberculosis is further aggravated by the alarming increase in human immunodeficiency virus infection as well as the emergence of drug-resistant strains of *Mycobacterium tuberculosis* (16, 18). One of the best prognoses for tuberculosis comes with early diagnosis of the infection and immediate implementation of appropriate treatment regimens. The diagnosis of the majority of TB cases in developing countries like India relies on acid-fast staining of sputum or positive cultures of *M. tuberculosis* in conjunction with assessment of clinical symptoms and radiographic evidence (6, 29, 38). However, these evaluations are usually expensive, tedious, and time-consuming. The most common method employed for detection of *M. tuberculosis* infection is the purified protein derivative (PPD) or tuberculin skin test, but PPD is a crude and poorly defined mixture of mycobacterial antigens, many of which are shared with proteins from the vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) and from nontuberculous environmental mycobacteria (20, 22). Therefore, the clinical relevance of the tuberculin test with PPD is not highly reliable (17, 30).

Although several new and rapid tests for the diagnosis of TB have been developed in recent years (7, 25, 33), they must be performed in laboratories and involve costly equipment and reagents. Further, most of the antigens in these tests have poor sensitivity and specificity to diagnose TB cases with smear-negative sputum samples and are not yet considered standard practice (4, 8). A serological test to detect antibodies to *M. tuberculosis* has the immense potential to make a diagnostic test for TB optimal and low-cost in developing countries, especially under field conditions (10). In recent years, numerous *M. tuberculosis* antigens that are capable of generating specific antibody titers in TB patients have been identified, but no single antigen appears to be ideal for serodiagnostic assays (13, 21, 26). Therefore, identification of an appropriate *M. tuberculosis* antigen suitable for serodiagnosis that can offer high specificity, ease of detection, and sensitivity that can distinguish active tuberculosis patients from BCG-vaccinated controls is highly desirable for developing suitable control measures and early treatment of the disease.

A significant portion (~10%) of the *Mycobacterium tuberculosis* genome encodes two unique protein families, the PE and PPE families, with no known apparent functions (11). The PE/PPE genes are expressed upon various environmental cues during infection, and many of the PPE proteins have been found to be strongly immunogenic (10, 12, 15, 32). Recently, studies have shown that mycobacterial PPE antigen Rv1168c (PPE 17) is associated with ESAT-6 gene cluster region 5 (ESX-5) (19), which is predicted to encode a novel secretory apparatus (1, 19). It has also been shown that this cluster is conserved among the various pathogenic mycobacteria, but not in the saprophytic species *Mycobacterium smegmatis* (1). Further, a BLAST analysis suggested that no genes that are strongly homologous to Rv1168c are present in the non-TB mycobacterial species that have been sequenced. The absence of homologues in other mycobacterial species, such as *Mycobacterium avium*, makes this protein a potential candidate for serological diagnosis of TB, which is further corroborated by its high antigen index, as calculated with the Kyte-Doolittle algorithm. Interestingly, the recent microarray and proteome

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